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(54) Title: SOLUBLE SINGLE CHAIN T CELL RECEPTORS

(57) Abstract

A fusion protein, comprising a carrier protein connected by a peptide tether to a single chain T cell receptor molecule, consisting of a Vα segment linked to a Vβ segment of the T cell receptor, is disclosed. Also disclosed is a soluble single chain T cell receptor molecule with a conformation that is essentially functionally indistinguishable, based upon reactivity to clonotype-specific antibodies, from that appearing on the surface of T cells. The invention also concerns nucleic acid fragments encoding the fusion protein, expression vectors comprising a nucleic acid fragment encoding the fusion protein, host cells containing such expression vectors, and antibodies to the single chain T cell receptor or to the fusion protein. The invention further pertains to methods of isolating and purifying the fusion proteins, as well as isolating and purifying soluble, single chain T cell receptors. In addition the invention pertains to various uses of soluble TCR fusion protein and isolated single chain TCR. The proteins can be used in molecular assays designed to measure their binding to ligands, including MHC/HLA-peptide antigen complexes or TCR-specific antibodies. Such assays are useful for the detection of agents that block the TCR-ligand interaction. The soluble TCR proteins can also be used to immunize animals, including humans, to produce TCR-specific antibodies. In addition, either in their native or denatured conformation the proteins can be used to vaccinate animals, including humans, in order to suppress the immune response of T cells bearing TCR that share antigenic epitopes with the vaccinating protein.

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# SOLUBLE SINGLE CHAIN T CELL RECEPTORS

## Background of the Invention

The T cell receptor (TCR) is a clonally expressed cell surface protein of T lymphocytes which mediates recognition of foreign antigens. It is composed of six polypeptide chains, two of which form a heterodimer and are unique to any given clonal T cell line. Four polypeptides ($\alpha$, $\beta$, $\gamma$, $\delta$) form two different heterodimers ($\alpha$:$\beta$ and $\gamma$:$\delta$); the $\gamma$:$\delta$ heterodimer appears earlier than the $\alpha$:$\beta$ heterodimer in the development of an organism (Davis, M. M. and P. J. Bjorkman, *Nature 334*:395-402 (1988)). The amino terminal half of the $\alpha$ and $\beta$ (or $\gamma$ and $\delta$ depending on the T cell subtype) chains which comprise the TCR are known as the variable (V) regions because the unique specificity of the TCR is a reflection of the marked amino acid sequence diversity of these regions. This sequence diversity determines the specificity of the TCR, enabling recognition of a vast array of protein fragments, or epitopes, presented by the "restricting element", the major histocompatibility complex (MHC) (known in humans as the HLA complex) class I and class II proteins (Germain, R.N., *Cell 76*:287-299 (1994)). Recognition by the TCR of antigen in the context of MHC (or HLA) molecules triggers T cell activation, thus initiating the immune response.

The sequences of the TCR $\alpha$- and $\beta$-chain variable regions are encoded by gene segments that undergo somatic recombination to form complete transcriptional units during T cell development (Davis, M.M. and P. Bjorkman, *Nature 334*:395-402 (1988)). Because rearrangements of the V and J segments of the $\alpha$-chain family and the V, D, and J segments of the $\beta$-chain family occur independently in each developing T cell, the TCR repertoire of antigen-binding

specificities is expressed clonally. This has been demonstrated by the observation that the sequences of the functionally rearranged TCR genes from independently derived T cell clones encode TCR α- and β-chains with different primary amino acid sequences (see, for example, Fink, P.J. *et al.*, *Nature 321*:219-226 (1986)).

Many groups have tried different approaches for producing soluble paired variable regions of the α/β TCR: (i) as variable regions connected by a polypeptide linker to create single chain (sc) TCR molecules; (ii) as fusions with immunoglobulin kappa light chains; and (iii) as phosphotidylinositol-linked heterodimers on the surface of cells in tissue culture.

The single chain TCR (scTCR) approach outlined by Novotny *et al.* (*PNAS USA 88*:8646-8650 (1991)) relies on expression of the scTCR α/β in *E. coli*. This expression system offers efficient production of protein in high yields; however, much of the bacterially-derived scTCR is aggregated, improperly folded and insoluble. Refolding of purified and denatured recombinant proteins is often an inefficient process because the denatured scTCR is highly insoluble and prone to aggregation or precipitation when undergoing refolding. Moreover, the apparent low solubility of the scTCR as expressed in bacteria in aqueous solvents further reduces the yield following renaturation.

There have been many attempts to express TCR α- and β-chains in eukaryotic cells (Traunecker, A., *et al.*, *Immunol. Today 10*:29 (1989). When the genes were initially cloned into appropriate expression vectors and transfected into cultured mammalian cells, expression of TCR α/β heterodimers could not be obtained in the absence of coexpression of γ, δ, ε, and ζ chains; that is, the other members of the group of proteins that together form the CD3 complex. If the α- and β-chains were not assembled into a

CD3 complex, the protein was degraded in the endoplasmic reticulum (Wileman *et al., Cell Regulation 1*:907-919 (1990)). It was later determined that a signal for degradation resided in the transmembrane region of the TCR α and β polypeptide chains (Wileman *et al., Cell Regulation 1*:907-919 (1990); Wileman *et al., J. Cell Biol. 110*:973-86 (1990); Bonnifacino *et al., Science 247*:79-84 (1990); Bonnifacino *et al., Cell 63*:503 (1990); Shin *et al., Science 259*:1901 (1993)).

It has also been shown that both α and β chain extracellular domains can be synthesized as soluble chimeric molecules with carboxy-termini derived from immunoglobulin molecules (Mariuzza and Winter; Gregoire, C. *et al. Proc. Natl. Acad. Sci. USA 88*:8077-8081 (1991); Gascoigne, N.R.J. *et al., Proc. Natl. Acad. Sci. USA 84*:2936-2941 (1987); Weber, S. *et al., Nature 256*:793-796 (1992)).

Phosphatidyl inositol membrane anchored α/β TCR heterodimers have been produced on the surface of CHO cells, and enzymatically released from the cell surface by phospholipase C treatment ((Lin, A.Y. *et al., Science 249*:677 (1990); Slanetz A.E. and Bothwell, A.L.M., *European Journal of Imm. 21*:179-183 (1991); however, small amounts of soluble TCR were produced, and the method is not practical for the production of milligram quantities.

Obtaining the unique portion (VαVβ) of the TCR in amounts sufficient for biochemical and immunological characterization, and in the correct conformation, is essential for developing a more complete understanding of the immune system. Furthermore, variable regions of TCR may provide drug targets that could potentially be specific for T cells involved in pathological mechanisms. Examples of T cell-mediated pathology in human diseases include: pancreatic β-cell destruction in insulin-dependent diabetes mellitus (IDDM), demyelination within the central nervous

system in multiple sclerosis, and graft rejection following allografting between HLA incompatible individuals. Production of the variable region of the TCR in soluble form is a prerequisite for determining the structure of the TCR involved in disease, and for constructing receptor-ligand assays for screening for TCR antagonists.

Summary of the Invention

The present invention concerns a polypeptide molecule (fusion protein) comprising a carrier protein connected by a peptide tether to a single chain T cell receptor (scTCR) molecule containing a Vβ segment connected by a peptide linker to a Vα segment of the TCR. After digestion of the peptide tether between the carrier protein and the scTCR, a soluble scTCR protein is produced which has a conformation essentially indistinguishable from that which appears uniquely on the surface of T cells. This soluble scTCR protein is biologically functional and does not require renaturation of the protein. The invention also concerns recombinant nucleic acid molecules comprising a defined sequence encoding the fusion protein, as well as expression vectors containing a nucleic acid sequence encoding the fusion protein, prokaryotic or eukaryotic host cells transformed or transfected with such expression vectors, and antibodies (either monoclonal or polyclonal) to the scTCR protein or to the fusion protein. The invention further pertains to methods of isolating and purifying the fusion protein, as well as methods of isolating and purifying the soluble scTCR protein.

In one embodiment of the invention, a fusion protein is constructed, wherein a scTCR is tethered to the *E. coli* periplasmic maltose binding protein (MBP) encoded by the MalE gene. In the fusion protein, MBP is referred to as the carrier protein. A plasmid can be constructed containing a recombinant gene which encodes a fusion

protein comprising a maltose binding protein of *E. coli* at the amino terminus, connected by a peptide tether to a single chain T cell receptor (scTCR) molecule in which the Vβ segment is connected by a peptide linker to the Vα segment. The Vβ segment can be connected to the peptide linker such that the linker joins the carboxy terminus of the Vβ segment to the amino terminus of the Vα segment; alternatively, the Vβ segment can be connected to the peptide linker such that the linker joins the amino terminus of the Vβ segment to the carboxy terminus of the Vα segment. The scTCR molecule can further contain a hexahistidine tag connected to the carboxy terminus of the scTCR. After transection and culture of the plasmids in host cells under conditions allowing replication, transcription and translation of the plasmid, the cells are lysed and the fusion protein is isolated through standard methods. A significant quantity of the scTCR is correctly folded, both before and after proteolytic digestion of the protease sensitive tether between the MBP and the scTCR. Further purification of the fusion protein can be conducted to minimize aggregation and maximize yield of properly folded scTCR.

Another embodiment of the invention pertains to methods of isolating and purifying the fusion protein and also the scTCR of the invention. The methods include the steps of: subjecting the fusion protein to a first cycle of amylose affinity chromatography, followed by nickel affinity chromatography, anticlonotypic immunoaffinity chromatography, size exclusion chromatography, and a second cycle of amylose affinity chromatography. This procedure yields purified MBP-scTCR fusion protein having a conformation that is functionally indistinguishable, based upon reactivity with clonotype-specific antibodies, from the conformation which appears on the surface of T cells (referred to herein as a "native-like" or "native"

conformation). Purified scTCR can be obtained from purified MBP-scTCR fusion protein by subjecting the purified fusion protein to thrombin digestion, followed by nickel affinity chromatography.

The scTCR molecules of the invention can be used to detect and analyze the peptide and MHC/HLA molecular constituents of TCR ligands. The scTCR can also be used for diagnostic purposes, such as for the detection of T cells with pathogenic properties. The scTCR can additionally be used in functional, cellular and molecular assays, and in structural analyses, including X-ray crystallography, nuclear magnetic resonance spectroscopy, and computational techniques, designed to identify TCR antagonists or agents that inhibit the interaction between TCR and MHC/HLA molecules complexed with antigenic peptides. Similar techniques can be performed to screen for agents capable of blocking the interaction of TCR with TCR specific antibodies. The scTCR can additionally be used *in vivo*, in order to compete with pathogenic T cells; or to immunize mammals, particularly humans, against TCR structures that occur on the surface of T cells which perform pathogenic or otherwise undesirable functions. The TCR-specific antibodies raised against scTCR can be used in therapeutic strategies that are designed to regulate immune responses *in vivo* by either inhibiting or eliminating specific antigen-recognition by T cells. By selecting antibodies that recognize defined epitopes of the TCR, a restricted subset, or a clone of T cells involved in a disease or medically undesirable immune response, can be targeted. The antibodies can be unmodified, or can also be linked to cytotoxic drugs, toxins, enzymes or radioactive substances.

## Brief Description of the Drawings

Figure 1 depicts a schematic diagram of the MBP-scTCR fusion protein of the invention.

Figure 2 depicts the nucleic acid (SEQ ID NO. 1) and amino acid (SEQ ID NO. 2) sequences of the maltose binding protein (MBP). The SacI restriction site used for cloning is overlined and labeled at the end of the sequence.

Figure 3 depicts the nucleic acid (SEQ ID NO. 3) and amino acid (SEQ ID NO. 4) sequences of the Vβ and Vα regions of the D10 TCR joined by a linker. These nucleic acid sequences were incorporated into the MBP-D10 scTCR fusion protein.

Figure 4 depicts the nucleic acid (SEQ ID NO. 5) and amino acid (SEQ ID NO. 6) sequences of the Vβ and Vα regions of the B10 TCR joined by a linker. These nucleic acid sequences were incorporated into the MBP-B10 scTCR fusion protein.

Figure 5 is a depiction of the scheme for the purification of monomeric MBP-scTCR.

Figure 6 is a depiction of the scheme for the purification of scTCR.

Figure 7 is a graphic representation of the separation of monomeric fusion protein by size exclusion chromatography.

Figure 8 is a graphic representation of size exclusion chromatography of isolated D10 scTCR.

Figure 9 is a graphic representation of the estimation of the molecular weight of D10 scTCR using size exclusion chromatography.

Figure 10 is a graphic representation of data from electrospray mass spectrometry of a sample of 100 μg D10 scTCR.

Figure 11 is a graphic representation of data from electrospray mass spectrometry of a second sample of 100 μg D10 scTCR.

Figure 12 is a graphic representation of data from electrospray mass spectrometry of a third sample of 100 μg D10 scTCR.

Figure 13 is a graphic representation of the circular dichroism spectrum of D10 single chain TCR.

Figure 14 is a graphic representation of the triggering of D10 T cell proliferation by the D10 TCR clonotype-specific monoclonal antibody (mAb) 3D3, and blockage of the triggering by the MBP D10-scTCR fusion protein.

Figure 15 is a graphic representation of the ability of scTCR to block the antigen-specific activation of D10 T cell proliferation.

Figure 16 is a graphic representation of cytofluorimetry demonstrating D10 cell-specific staining with an AKR mouse antiserum raised against the D10 scTCR.

Figure 17 is a graphic representation of the proliferative response of D10 T cells to an antiserum raised against the D10 scTCR in an AKR mouse.

Figure 18 is a graphic representation of the specificity of antisera to soluble TCRs.

Figure 19 is a graphic representation of the proliferative response of D10 T cells to the monoclonal antibody 3E9G2. Squares = background; circles = 3E9G2; diamonds = mAb 3D3 (control).

Figure 20 is a graphic representation demonstrating the specific binding of mAb 3E9G2 to D10 scTCR.

Figure 21 is a graphic representation demonstrating the blocking of mAb 3E9G2 binding to D10 scTCR by mAb Vα2.

Figure 22 is a graphic representation demonstrating the blocking of 3E9G2 binding to D10 scTCR by mAb 3D3.

Figure 23 is a graphic representation demonstrating that mAb Vβ8 does not block binding of mAb 3E9G2 to D10 scTCR.

Figure 24 is a graphic representation demonstrating that mAb 3E9G2 does not block binding of mAb Vβ8 to D10 scTCR.

Figure 25 is a graphic representation of immunomodulation of the B10.A mouse response to cytochrome C by vaccination with the MBP-B10 scTCR fusion protein in complete Freund's adjuvant.

Figure 26 is a graphic representation of the B10.A mouse response to cytochrome C after vaccination with complete Freund's adjuvant alone.

Detailed Description of the Invention

The present invention concerns a polypeptide molecule, or fusion protein, comprising a single chain T cell receptor (scTCR) tethered at its amino terminus to the carboxy terminus of a carrier protein, such as maltose binding protein (MBP). The scTCR comprises a Vα fragment joined by a peptide linker to a Vβ fragment. A hexahistidine tail can be joined to the carboxy terminus of the scTCR. The fusion protein is soluble when purified; moreover, the fusion protein reacts with anti-clonotypic antibodies that are specific for the correctly folded conformation of the TCR. As described in detail below, soluble scTCRs can be produced that are in a native-like conformation that is functionally equivalent to the cell surface TCR determinant that is unique to a particular clonal line of T cells. The invention also pertains to methods of isolating and purifying the fusion proteins and the scTCR after cleavage of the fusion proteins with enzymes specific for peptide linkages within the tether. These methods have been designed to enhance recovery of protein that is folded in a native-like conformation.

Several steps are taken to generate scTCRs. First, nucleic acid fragments bearing gene sequences for the Vα and Vβ segments of the TCR of interest are isolated. The

nucleic acid fragments can be DNA or cDNA molecules that are isolated by known methods. For example, synthetic oligonucleotide primers corresponding to portions of the Vα and Vβ gene sequences can be used in the polymerase chain reaction (PCR) to amplify DNA or cDNA prepared from T cells bearing the TCR of interest. The nucleic acid fragments encoding the Vα and Vβ fragments are joined together by a nucleic acid fragment encoding a peptide linker utilizing known methods, such as by cloning the nucleic acid fragments encoding the Vα and Vβ segments into a vector containing the nucleic acid fragment encoding the peptide linker. The nucleic acid sequence for the peptide linker between the TCR V region sequences can be generated by known methods (Aota, S., *et al.*, *Nucl. Acids Res. 16 Suppl:* R315-R402 (1988); Pantoliano, M.W., *et al.*, *Biochemistry 30:*10117-25 (1991)). The nature of the amino acids in the peptide linker used to join the TCR V region fragments can be critical to imparting the proper three dimensional conformation to the scTCR molecule. In addition to a polyglycine containing structure, the linker benefits from charged residues which can aid solubility and stabilize interactions between the complementary faces of the two V region domains of the scTCR. To determine whether a particular linker allows the scTCR to form the native conformation, the scTCR is tested for the native conformation utilizing the methods described by Engel *et al.* (*Science 256*:1318 (1992)). If desired, the plasmid encoding the fusion protein can be modified so as to add a hexahistidine tail to the carboxy terminus of either the Vα or Vβ segment, depending on the order of the gene segments in the construct.

The nucleic acid fragment encoding the scTCR is joined to a nucleic acid fragment encoding a peptide tether that is joined to a nucleic acid fragment encoding a carrier protein. The peptide tether is designed to allow

access to a unique cleavage site, such as an enterokinase site (having the amino acid sequence DYKDDDDK (SEQ ID NO. 7), which is also known herein as a "FLAGG" sequence), a Factor Xa site (having the amino acid sequence IEGR (SEQ ID NO. 8)), or a thrombin cleavage site (cleaving four amino acids into the amino acid sequence LVPRGS (SEQ ID NO. 9). The carrier protein can be any protein which allows the fusion protein to remain soluble in aqueous buffers, and which lacks cysteine residues or disulfide bonds. In one embodiment, the maltose binding protein (MBP) of *E. coli* is used. Coding regions from *Staphylococcus aureus* protein A can also be used. The nucleic acid fragment encoding the scTCR is joined with the nucleic acid fragment encoding the peptide tether and the carrier protein. For example, the nucleic acid fragment encoding the scTCR can be inserted into an expression vector containing the nucleic acid fragment encoding the peptide tether and the carrier protein. This generates a recombinant vector encoding the fusion protein (the fusion protein vector). Alternatively, the nucleic acid fragments encoding the carrier protein and the tether can be attached to the nucleic acid fragment encoding the scTCR before insertion of the construct into an expression vector. A representative expression vector which contains the nucleic acid sequence of MBP is the vector pPR998 developed by P. Riggs (*Current Protocols in Molecular Biology*, (Ausebel, F.M. *et al.*, eds.), Greene Assoc./Wiley Interscience, New York, section 16.6, 1992; the vector is available from New England Biolabs, Beverly, MA, USA).

Figure 1 depicts a schematic map of a representative fusion protein which utilizes MBP as the carrier peptide (MBP-scTCR fusion protein). The nucleic acid (SEQ ID NO. 1) and amino acid (SEQ ID NO. 2) sequences of the MBP are depicted in Figure 2. The nucleic acid and amino acid sequences of two exemplary fusion proteins are depicted in

Figure 3 (MBP-D10 scTCR) and Figure 4 (MBP-B10 scTCR). From left (amino terminus) to right (carboxy terminus) in Figure 1, SS is the signal sequence, of approximately 26 amino acids; MBP is the maltose binding protein (approximately 370 amino acids); T is the thrombin cleavage site (having the amino acid sequence LVPR); Vβ is the variable region of the β chain (approximately 110 amino acids); L is the linker (approximately 26 amino acids); Vα is the variable region of the α chain (approximately 114 amino acids); and HH is the optional hexahistidine tag. In the recombinant gene encoding the MBP-scTCR fusion protein, nucleotide sequence encoding the tether typically begins from a 5′ *sacI* site and extends downstream to the thrombin cleavage site. The Vβ coding region typically begins immediately 3′ of the sequence encoding the site of thrombin cleavage, and extends downstream to a *BamHI* site. Sequence encoding the linker between the Vβ and Vα regions of the TCR typically extends from the *BamHI* site to a downstream *NarI* site. The linker between the Vα and Vβ domains shown in Figure 3 is called the 3XG linker. The Vα coding region of the TCR typically begins immediately 3′ of the *NarI* site and extends downstream to a stop codon and a *HindIII* site at the 3′ end. In the example of Figure 3, sequence encoding a hexahistidine tail has been incorporated immediately 5′ of the *HindIII* site.

In one particular embodiment of the invention, Vα and Vβ cDNA sequences from either the D10 or B10 T cell lines are synthesized using PCR, and then sequentially cloned into a vector encoding a linker to connect the Vα and Vβ sequences. The recombinant gene thus generated encoding the scTCR is then cloned into the vector pPR998 encoding MBP under a hybrid trp/lac promoter.

The fusion protein is expressed in an appropriate vector and host system. A host cell is transformed or transfected with the fusion protein vector for replication,

transcription and translation. The host cell can be prokaryotic. Gram negative bacterial strains, such as *Escherichia coli*, as well as gram positive bacterial strains, such as *Staphylococcus aureus*, can be used. Alternatively, eukaryotic cells of mammalian or insect origin, or yeast such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, can be used. In a preferred embodiment, *E. coli*, and particularly the strains XL1Blue (Stratagene, LaJolla, CA) or BL21 (Novagen, Madison, WI), are used as hosts. Alternatively, a phage display format can also be used to produce the fusion protein (Scott, J.K. and Smith, G.P., *Science* 249:386-390 (1990); Barbas, C.F., *et al.*, *PNAS USA* 88:7978-7982 (1991)). The fusion protein vectors of the present invention can be introduced into host cells by various methods known in the art. For example, transection of host cells with fusion protein vectors can be carried out by electroporation. Other methods can also be employed for introducing fusion protein vectors into host cells; calcium phosphate, calcium chloride or ruthenium chloride mediated-transection, or other techniques, some involving membrane fusion, can be used.

Once a fusion protein vector has been introduced into appropriate host cells, the host cells are cultured under conditions permitting expression of large amounts of scTCR fusion protein. The expressed scTCR fusion proteins can be purified to homogeneity from host cell lysates by known methods, such as by affinity chromatography and standard biochemical techniques. The fusion proteins can be further purified to eliminate aggregation and maximize yield of fusion protein with a native conformation, such as by nickel affinity chromatography. If desired, the scTCR can be freed from the carrier protein through specific proteases, such as thrombin, which cleaves a unique site in the tether. Such cleavage results in the generation of a

soluble scTCR with a native-like conformation. The scTCR can be assayed immunologically using conformation sensitive immunoassays that are performed on the sample either before or after proteolytic digestion at the tether to liberate free scTCR. The scTCR can be tested for the presence of the native conformation utilizing the methods described by Engel *et al.* (*Science 256*:1318 (1992)). These workers transfected the rat basophilic leukemia line RBL-2H3 with recombinant genes encoding the TCR extracellular domains linked to the transmembrane segment and cytoplasmic tail of the zeta chain. The transfected cells expressed heterodimeric TCR on the cell surface. This TCR could appropriately recognize the stimulatory peptide bound to the I-$E^k$ MHC class II molecule, resulting in MHC-restricted activation of the RBL cells.

Thus, it is explicitly proposed that in the absence of appropriate conformation-sensitive, clonotype-specific antibodies, a single chain TCR might be produced in RBL cells as a membrane-bound fusion protein attached via its carboxy terminus to the transmembrane and intracellular domains of CD3 zeta chain. It is suggested that adjustments to the linker leading to correctly folded scTCR might be monitored by measuring activation of these RBL cells in a manner similar to Engel *et al.* (1992). In such an experiment, correct binding of scTCR to MHC/peptide will result in activation of the RBL cells. Linker sequences that do not permit the scTCR to adopt the correct conformation will not lead to significant activation of the RBL cells. Thus linker sequences can be varied and selected for their ability to allow correct folding of the TCR.

The invention also pertains to methods of isolating and purifying the fusion protein and scTCR described above. After the fusion protein is produced in appropriate host

cells, the host cells are lysed and the lysed cells are fractionated by centrifugation. The supernatant, which contains the fusion protein, is subjected to a first cycle of amylose affinity chromatography, generating "amylose pure fusion protein". After purification by amylose affinity chromatography, the fusion protein is subjected to nickel affinity chromatography designed to refold the fusion protein into the native-like conformation. Fusion protein which has been subjected to nickel affinity chromatography is referred to herein as "refolded" fusion protein, and the process of nickel affinity chromatography referred to as "refolding". The "refolding" process enhances (increases) the amount of fusion protein that is in the desired conformation. The refolded fusion protein is subjected sequentially to immunoaffinity chromatography, size exclusion chromatography, and a second cycle of amylose affinity chromatography. The resultant product is isolated and purified MBP-scTCR that is in a native-like conformation. The fusion protein consists of two domains: carrier protein MBP and $V\alpha/V\beta$ (or $V\beta/V\alpha$) of scTCR. Each is judged to be correctly folded by its ability to react with a ligand that is only recognized when the relevant domain is in its native conformation. For MBP the ligand is amylose, and for scTCR, an antibody whose epitope is conformational; that is, an epitope which is dependent on the pairing and folding of the $V\alpha$ and $V\beta$ segments in the native conformation. The series of steps is depicted in Figure 5. The MBP-scTCR that is isolated and purified by the above method can be further processed to isolate soluble scTCR. The purified MBP-scTCR is digested with thrombin to yield the MBP and scTCR as separate molecules which can be fractionated by nickel affinity chromatography. Soluble scTCR is bound, and then eluted from the nickel column as monomeric, soluble scTCR in a

native conformation. The series of steps is depicted in Figure 6.

The scTCR and MBP-scTCR of the invention can be used to derive TCR structures for identification of TCR antagonists or agents that inhibit the interaction between the TCR and MHC/HLA molecules complexed with antigenic peptides. TCR structures can be applied in rational drug design using computational techniques. TCR structural information derived from one scTCR can be used to deduce general rules concerning the whole class of TCR proteins or certain subsets thereof, thereby aiding in the identification of inhibitory compounds. Structural information concerning one particular scTCR can be used to devise highly specific inhibitors for a particular T cell clone. Structural information from one scTCR can be obtained by standard methods, including information obtained from X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, or biochemical or biophysical investigation of the interaction of the scTCR with ligands such as MHC/HLA molecules complexed with antigenic peptide or superantigen, or TCR-specific antibodies. Superantigens are proteins that share the ability to bind to human and mouse HLA/MHC Class II proteins to form a ligand complex for the Vβ segment of the TCR. Because it binds to Vβ segments belonging to particular families, a superantigen-HLA/MHC Class II complex can stimulate many more T cells than a complex of a particular Class II molecule and an antigenic peptide. Superantigens are represented by the Staphylococcal enterotoxins and Streptococcal toxins (Marrack, P. and Kappler, J., *Science* 248:705-711, 1990), and by proteins encoded by endogenous retroviruses (for example, Woodland, D.L. *et al.*, *Nature* 349:529-530 (1991)). Once structural information concerning one TCR is obtained, it can then be used to solve the crystallographic structure of other TCR by molecular replacement techniques.

Consequently, structural coordinates of any TCR can be used in the determination of the structure of TCR of pathological importance in mammals, particularly humans.

The scTCR and MBP-scTCR of the invention can additionally be utilized in assays to screen for agents that inhibit the interaction of TCR with: 1) complexes formed between MHC/HLA molecules and antigenic peptides or superantigens (referred to herein collectively as antigens), and 2) TCR specific antibodies, including but not limited to anti-clonotypic antibodies. Such agents include TCR blockers or antagonists, MHC/HLA blockers or antagonists, and molecular mimics of the TCR ligands. To conduct the assay for agents that inhibit the interaction of TCR with the complexes formed between MHC/HLA molecules and antigenic peptides or antigens, a sample of isolated and purified scTCR is incubated with the MHC/HLA molecules and antigenic peptides or superantigens of interest, under conditions that allow the scTCR to interact with the MHC/HLA molecules and antigenic peptides/superantigens. This sample is the control sample. A second sample (the test sample) identical to the control sample except that it is exposed to the agent to be tested, is also incubated under the same conditions. Both the control sample and the test sample are then evaluated to determine the level of interaction of TCR with the complexes formed between the MHC/HLA molecules and antigenic peptides or superantigens of interest. If less interaction occurs in the presence of the agent to be tested (in the test sample) than in the absence of the agent to be tested (in the control sample), then the agent is an inhibitor of the interaction between TCR and the complexes formed between the MHC/HLA molecules and antigenic peptides or superantigens of interest. To conduct the assay for agents that inhibit the interaction of TCR with TCR specific antibodies, an assay similar to that described above is conducted, using a sample of

isolated and purified scTCR that is incubated with the TCR specific antibody of interest as the control sample. Less interaction between the scTCR and the antibody in the presence of the agent to be tested, than in the absence of the agent to be tested is indicative that the agent is an inhibitor of the interaction between TCR and the TCR specific antibody of interest. For example, the antibody 3D3 can be used for D10 scTCR and the antibody 8G2 for B10 TCR.

The scTCR or the fusion protein MBP-scTCR of the invention can also be used to detect the MHC/HLA molecular constituents of TCR ligands using molecular assays. Recombinant, soluble forms of MHC/HLA molecules can be immobilized on a solid support. Synthetic and/or naturally occurring peptides can be incubated with the MHC/HLA molecules to form complexes that can be investigated for their ability to bind scTCR or the MBP-scTCR added in the solvent phase. Binding of the receptor proteins can be detected utilizing TCR-specific antibodies and standard ELISA, or by surface plasmon resonance using the BIAcore™ (Pharmacia LKB Biotechnology, Inc., New Jersey) biosensor system (Fagerstam, L.*Tech. Prot. Chem.* 2:65-71 (1991); Malmqvist, M., *Current Biology* 5:282-286 (1993)). Identification of ligands recognized by T cells that are involved in disease states, such as those involved in the destruction of pancreatic β-cells in insulin-dependent diabetes mellitus (IDDM), would allow the establishment of cellular or molecular screening assays for agents that block activation of pathogenic T cells by interference with the binding of the T cell receptor to its ligand. Such assays would be conducted in a similar manner to the assays described above: a sample of isolated and purified scTCR of interest (i.e., scTCR that has a native-like conformation, generated by the methods described above) and its ligand is incubated under conditions that allow

interaction between the scTCR and its ligand; a second sample of scTCR and ligand is exposed to the agent to be tested and incubated in a similar manner. The level of interaction between the scTCR and ligand is then examined; a lower level of interaction in the presence of the agent than in the absence of the agent is indicative of the ability of the agent to block activation of the scTCR, and thus to block activation of the pathogenic T cells. Agents that could block activation of pathogenic T cells include antibodies to T cell receptors, such as those described below.

The scTCR of the invention can also be used to generate antibodies, either monoclonal or polyclonal, using standard techniques. The term "antibody", as used herein, encompasses both polyclonal and monoclonal antibodies, as well as mixtures of more than one antibody reactive with scTCR (e.g., a cocktail of different types of monoclonal antibodies reactive with scTCR). The term antibody is further intended to encompass whole antibodies and/or biologically functional fragments thereof, chimeric antibodies comprising portions from more than one species, humanized antibodies and bifunctional antibodies. Biologically functional antibody fragments which can be used are those fragments sufficient for binding of the antibody fragment to scTCR. Once the antibodies are raised, they are assessed for the ability to bind to scTCR. Conventional methods can be used to perform this assessment.

The chimeric antibodies can comprise portions derived from two different species (e.g., a constant region from one species and variable or binding regions from another species). The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as single contiguous proteins using genetic engineering techniques. DNA encoding the

proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed as contiguous proteins.

Monoclonal antibodies (mAb) reactive with scTCR can be produced using somatic cell hybridization techniques (Kohler and Milstein, *Nature 256*: 495-497 (1975)) or other techniques. In a typical hybridization procedure, a crude or purified scTCR protein, or peptide derived from the scTCR protein, can be used as the immunogen. An animal is immunized with the immunogen to obtain anti-scTCR antibody-producing spleen cells. The species of animal immunized will vary depending on the specificity of mAb desired. The antibody producing cell is fused with an immortalizing cell (e.g., myeloma cell) to create a hybridoma capable of secreting anti-scTCR antibodies. The unfused residual antibody-producing cells and immortalizing cells are eliminated. Hybridomas producing desired antibodies are selected using conventional techniques and the selected hybridomas are cloned and cultured.

Polyclonal antibodies can be prepared by immunizing an animal in a similar fashion as described above for the production of monoclonal antibodies. The animal is maintained under conditions whereby antibodies reactive with scTCR are produced. Blood is collected from the animal upon reaching a desired titer of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum can optionally be further separated into fractions of particular types of antibodies (e.g., IgG, IgM).

The antibodies of the invention can be used to detect T cells with pathogenic properties in mammals, including humans. To detect pathogenic T cells, a sample of lymphocytes is incubated with antibodies to the scTCR of interest (the scTCR that has a native-like conformation,

generated by the methods described above). Interaction between the lymphocytes and the antibodies is assessed; the presence of interaction between the lymphocytes and the antibodies is indicative of the presence of pathogenic T cells. The lymphocytes can be obtained, using standard techniques, from peripheral blood, bodily fluids (including cerebrospinal fluid, and synovial fluid), and lymph nodes, or spleen or other tissue biopsy specimens. Analysis of the lymphocytes can be performed before or after *in vitro* culture of the lymphocytes.

The antibodies of the invention can also be used to deplete T cells or inhibit T cell activation *in vivo* in mammals, including humans. Therapeutic regimens can be designed in which antibodies are administered, using standard methods, in order to inhibit antigen recognition, by binding to T cell surface TCR and thereby sterically blocking the interaction between the variable region of the TCR and the specific complex of antigenic peptide and MHC molecule. Alternatively, or in addition, the complexes formed between the TCR-specific antibodies and the cell surface TCR can deplete T cells by utilizing accessory elements of the immune system that destroy the antibody-bound T cell. It is anticipated that the Fc region of antibodies bound to TCR on the T cell surface will engage and activate cytotoxic mechanisms mediated by the complement system, macrophages, monocytes, or antibody-dependent cytotoxic cells. The efficiency of T cell depletion can be enhanced by administering TCR-specific antibodies that are covalently conjugated to a cytotoxic or anti-metabolic agent, such as toxins of microbial or synthetic origin, including peptide toxins or polypeptides related to toxins (Frankel, A.E., *J. Biol. Response Mod.* *4*:437-446 (1985)); enzymes; radioactive substances; or cytotoxic drugs (Hawkins, R.E., *et al.*, *British Medical Journal* *305*:1348-1352 (1992)). In applications of TCR-

specific antibodies *in vivo* as immune response modifiers, immunoregulators or immunosuppressors, the selection of antibodies with defined specificity allows targeting of either the whole T cell population, or a defined T cell sub-population, within an individual animal or human. For example, antibodies specific for a clonotypic epitope would target only the members of a single T cell clonotype, whereas antibodies specific for a Vβ family-specific epitope would target all the T cell clones bearing TCR utilizing Vβ-segments belonging to that particular family. Only those T cells involved in a particular disease or medically undesirable immune response would be targeted for modulation or elimination; the majority of T cells involved in the maintenance of immunity against infectious agents would be spared. The antibodies to the TCR are administered to a mammal in a therapeutically effective amount, which is the amount of the antibody that is necessary to inhibit the activation of, deplete or eliminate the pathogenic T cells.

The scTCR of the invention can also be used *in vivo* in mammals, including humans, to compete with pathogenic T cells for their specific MHC/HLA class II associated peptide antigen. In this manner, the scTCR can be used to deplete antigen such that the activation of the pathogenic T cells would be reduced or eliminated *in vivo*. Pathogenic T cells of interest include those which are involved in pancreatic β-cell destruction in insulin-dependent diabetes mellitus (IDDM), demyelination within the central nervous system in multiple sclerosis, and graft rejection following allografting between HLA incompatible individuals. The scTCR are administered to a mammal in a therapeutically effective amount, which is the amount of the scTCR that is necessary to reduce or eliminate the activation of pathogenic T cells.

The scTCR or MBP-scTCR of the invention can also be used to immunize mammals, including humans, against TCR antigenic structures that occur on the surface of T cells which perform pathogenic or otherwise undesirable functions (the "targeted T cells"), such as graft rejection following transplantation. Such T cells can be identified in samples of peripheral blood, or in biopsy specimens taken from lymphoid organs or sites of inflammation. Lymphocytes in a sample are purified and investigated *in vitro* for their ability to make a T cell dependent proliferative response to the relevant MHC/HLA associated antigenic epitope. The T cells that undergo cell division can be established *in vitro* as lines or clones from which TCR genes can be cloned and used to produce scTCR and MBP-scTCR by the recombinant DNA technology described herein. TCR antigenic structures include clonotypic epitopes, V$\alpha$ or V$\beta$ family-specific epitopes, conformational epitopes, and linear epitopes. Immunization against TCR antigenic structures that occur on the surface of the targeted T cells inhibits the activity of the targeted T cells, thereby reducing the pathogenic or undesirable effects of the targeted T cells. To immunize a mammal, the scTCR are administered to a mammal in an effective amount, which is the amount of the scTCR that is necessary to inhibit the activation of, deplete or eliminate the targeted T cells.

Administration of scTCR or antibody to TCR, whether it be for the reduction, depletion or elimination of the activation of pathogenic T cells, or for immunization, can be in the form of a single dose, or a series of doses separated by intervals of days or weeks. The term "single dose," as used herein, can be a solitary dose, and can also be a sustained release dose. The scTCR or antibody can be administered subcutaneously, intravenously, intramuscularly, intraperitoneally, orally, by nasal spray or by inhalation, opthamologically, topically, via a slow-

release compound, or via a reservoir in dosage formulations containing conventional, physiologically-acceptable carriers and vehicles. Alternatively, a DNA fragment encoding the scTCR can be administered. The formulation in which the scTCR or the antibody is administered will depend in part on the route by which it is administered, and the desired effect.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention.

EXAMPLE 1: Production of MBP-scTCR from the Conalbumin-Specific T Cell Line D10 and the Cytochrome C-Specific T Cell Line B10

A. Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Oligonucleotides were purchased from the Midland Certified Reagent Co. (Midland, TX). Enzymes and the expression vector pPR998 were purchased from and used as suggested by New England Biolabs (Beverly, MA). The bacterial strain utilized was XL1Blue Stratagene (La Jolla, CA). Immobilon membrane and enhanced chemiluminescence detection system were purchased from and used as suggested by Amersham Inc. (Arlington Heights, IL). The D10 T cell clone was obtained from ATCC (Rockville, MD) as the subclone designated D10.G4.1 (ATCC TIB 224). The cloned rearranged $\alpha$ and $\beta$ genes for the D10 TCR, and the 3D3 hybridoma (Kaye, *et al.*, *J. Exp. Med.* *158*:836-856 (1983)) producing the D10 clonotype-specific antibody were obtained from A.L.M. Bothwell, Yale University, New Haven CT. Partial sequences of the D10 TCR V$\alpha$ and V$\beta$ gene segments have previously been published (Hong, S-C, *et al.*, *Cell* *69*:999-1009 (1992)). Similar

reagents were used for other experiments described below using the cytochrome C specific T cell line, B10 (Fink *et al.*, *Nature 321*:219 (1986)). The hybridoma cell lines 1F2 (Kubo, *J. Immun.* *142*:2736-2742 (1989)); RR8 (Jameson S., *et al.*, *J. Immun.* *146*:2010 (1991)), and 8G2 (unpublished, gift of S. Smiley and E. Reinherz) used in these studies, produce monoclonal antibodies specific for the Vα11 of the B10 TCR in its native form only (1F2), Vα11 of both native and denatured B10 TCR (RR8), and a clonotypic structure requiring both α and β chains (8G2) which is destroyed by denaturation.

B. Construction of DNA Sequence Encoding Single Chain T Cell Receptor Fusion Protein MBP-scTCR

Oligonucleotide primers corresponding to the amino- and carboxy-terminal regions of the Vα and Vβ cDNA sequences cloned from the D10 T cell line were synthesized. These primers were employed in a polymerase chain reaction (PCR) to produce Vα and Vβ region encoding fragments of DNA that were then cloned into an expression vector as gene cassettes. The PCR primers were added, together with the cloned template DNAs, to a reaction mixture containing all four nucleotide triphosphates at 0.125 mM each, 10 mM $MgCl_2$, 10 mM DTT in 10 mM Tris-HCl (pH 7.8). DNA polymerase (1.2 units) from *Thermophillus aquaticus* was added to begin the reaction which was overlayed with mineral oil and cycled 25 times between 94°C for 2 min., 55°C for 2 min, and 74°C for 2 min. This was carried out in a Perkin Elmer/Cetus Thermocycler and was concluded by a 7 min extension reaction at 74°C. The mineral oil was extracted with chloroform, and the polymerized nucleic acids were separated from the reaction mixture by gel filtration. The DNA was then restricted with the enzymes *Kpn*I and *Bam*HI in the case of the β chain, and *Nco*I and

*Xho*I for the α chain. These fragments were then sequentially cloned into a vector that encoded a linker that was to serve as the peptide chain connecting the carboxy-terminus of the V region of the β chain to the amino-terminus of the V region of the α chain of the D10 TCR. The nucleic acid sequence (SEQ ID NO. 3), and the encoded amino acid sequence (SEQ ID NO. 4), of the resultant chimeric molecule is shown in Figure 3. The enzyme thrombin cleaves between the sixth and seventh amino acids; the Vβ region extends from the seventh amino acid to the 118th amino acid, and is followed by the 27 amino acid linker, which is followed in turn by the Vα amino acid sequence (including the J region up to the constant region) extending from residues 146 to 257. Residues 258 to 263 are the hexahistidine tail. The synthetic chimera was sequenced to verify that it encoded the desired sequence of amino acids. Residues 11 and 255 in the wild type D10 were both altered to encode S (serine). The second amino acid after the removal of the signal sequence, which was an A in the wild type, has been substituted by an S so that thrombin would cleave more efficiently. The entire scTCR (Figure 3) was cloned into the vector pPR998 which encodes MBP under control of the hybrid trp/lac promoter. The junction between the tether coding region and the MBP gene is at a *Sac*I site located in the polylinker of the vector at the carboxy terminal coding region of MBP.

C. Expression and Purification of MBP D10-scTCR in *E. coli* Following Amylose Affinity Chromatography

*E. coli* Strain BL21 harboring the recombinant plasmid described above were grown to saturation overnight in yeast tryptone (YT) medium in a 5-liter fermentation vessel (Miller, T.H., *Exp. in Mol. Gen.* Cold Spring Harbor Laboratory Press 1972, p. 443). The cells were grown at 27°C to an optical density of 15-20 (monitored at 600-nM

wavelength) and were induced with 1 mM isopropyl β-D-thiogalactoside. After three hours of induction, the cells were harvested by centrifugation at 4500 rpm for 20 min. The cell pellets were either processed immediately or frozen at -70°C. Typically, the yield of cell pellet was 300 g/5L of harvested media.

300 g of frozen cell paste was thawed on ice and then resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, and 0.1 mM 4-(2-aminoethyl)benzene-sulfonylfluoride-HCl (AEBSF) at 10 ml/g wet weight of cell pellet. The cells were lysed by passing through a microfluidizer (Microfluidics Corporation, Newton, MA) at 15,000 psi. The lysed cells were then centrifuged at 9000 rpm for 90 minutes and the supernatant was filtered through a 0.4μ filter using a pellicon unit (Millipore, Bedford, MA). The filtered supernatant (3 L) was applied to a 400 ml XK50/30 (Pharmacia, Piscataway, NJ) amylose affinity column (New England Biolabs, Beverly, MA) at 4°C at a flow rate of 3 ml/min. The column was washed with 6 column volumes of wash buffer containing 50 mM Tris-HCl (pH 8.0) and 0.2 M NaCl. The bound material was subsequently eluted with wash buffer containing 10 mM maltose. The resulting material migrates as a single predominant species with apparent molecular size of 70 kDa on reducing sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The major species present in this preparation of MBP D10-scTCR appears, on nonreducing PAGE, to be present as non-disulfide linked monomers. Some of the scTCR forms aggregates and precipitates. After amylose affinity purification, the approximate yield is 5 mg MBP-scTCR/g cells.

D. Design and Modification of Linkers Between the MBP B10-scTCR

Using an approach similar to that described in section B, the B10 TCR genes were used in conjunction with appropriate oligonucleotide primers to produce DNA in a thermocycler that was then cloned into a plasmid that directed the synthesis in *E. coli* of a MBP B10-scTCR fusion protein. This material can then be produced in a manner similar to the MBP D10-scTCR described above in sections B and C. Interestingly, when the sequence of the linker joining the Vβ and Vα regions was changed by the addition of a string of eight mainly hydrophilic amino acids, DYKDDDDK (SEQ ID NO. 7; the "FLAGG" sequence), the conformation of the resulting material was altered, and anti-clonotypic antibody reactivity was observed where previously there was none detectable. Figure 4 depicts the nucleic acid sequence (SEQ ID NO. 5) and the amino acid sequence (SEQ ID NO. 6) of the cytochrome C-specific B10 TCR produced as scTCR. The enzyme thrombin cleaves between the sixth and seventh amino acids. The native Vβ region (up to the constant region) extends from the seventh amino acid to the 123rd amino acid; it is followed by the 27 amino acid linker region, which is, in turn, followed by the Vα sequence (including the J region up to the constant region) extending from residue 150 to 236. Residues 237 to 242 are the six terminal histidine residues. The second amino acid residue of the Vβ region after removal of the signal sequence is P in the wild type (residue 8); it has been substituted by S so that thrombin would cleave more efficiently. In a preferred embodiment, this scTCR is encoded downstream of a sequence encoding the tether linking it to the MBP gene (SEQ ID NO. 1; see Figure 2). The signal sequence which is not part of the mature protein is encoded within the first 30 amino acids. The linker between the Vα and Vβ domains shown here is called the 3XG/FLAGG linker, which consists of the 19 amino acid 3XG linker having the FLAGG sequence, DYKDDDDK (SEQ ID NO. 7),

inserted therein. The coding region is bounded by *Bam*HI and *Nar*I restriction sites. Samples of the MBP-B10 scTCR fusion protein, either undigested or digested with thrombin, were analyzed by the conformation sensitive assay described in Example 4(F) below. Fusion proteins were investigated with or without the eight amino acid FLAGG sequence inserted into the 3XG linker. Each sample was blotted onto duplicate membranes. One membrane was incubated in PBS at room temperature, and the other in PBS/2-ME at 100°C. The results indicated that the modified linker favored a conformation of the scTCR in which the Vα segment was correctly folded, as indicated by reactivity with the conformation sensitive antibody 1F2. The Vα and Vβ segments appeared to pair correctly in the presence of the modified linker, because the scTCR contained the epitopes recognized by the 8G2 antibody which is both conformation-sensitive and specific for the B10 TCR clonotype.

E. Construction of DNA Sequence Encoding Single Chain T Cell Receptor Fusion Protein MBP-scTCR$_{HH}$

Using methods such as those described above, a fusion protein comprising MBP-scTCR with a carboxy-terminal hexahistidine (HH) sequence was constructed. The carboxy-terminal extension of six histidine residues facilitated purification of the fusion protein, and also minimized isolation of MBP-scTCR with truncation of the carboxy terminus due to proteolytic digestion.

To construct plasmids encoding fusion proteins with additional histidine residues, the PCR reaction was used. Two synthetic oligonucleotides described below were used as primers, and plasmids that direct the synthesis of either MBP B10-scTCR or MBP D10-scTCR were used as templates. The PCR reaction products were isolated and cloned, using the

enzymes HindIII and EcoRI, into the vector pSP72 to create p3/566. This D10 plasmid, and p5/548 (a MBP B10-scTCR$_{HH}$ encoding plasmid) were then sequenced to verify the fidelity of the PCR reaction. The new fragments of DNA encoding the B10 and D10 Vα segments with hexahistidine tails were then used to replace the equivalent regions in the parent plasmids. Bacteria containing these plasmids were grown on an analytical scale and examined for correct protein expression prior to being grown on a large scale for biochemical purification of the fusion proteins.

The D10 α polyhistidine C terminal PCR primer used was 5′-CCCCAAGCTTCAATTAATGGTGATGGTGATGGTGATATGGGGACACA-GCCAGTCTGGTCCC-3′ (SEQ ID NO. 10). The D10 α N-terminal PCR primer was 5′-CGAATTCAGGCGCCCAGCAGCAAGTGAGACAAAGTCCCC-3′ (SEQ ID NO. 11). The B10 α polyhistidine C terminal PCR primer used was 5′-CCCAAGCTTTCATTAGTGATGGTGATGGTGATGGTACACCTTTAATATGGTCCCCT-GGCC-3′ (SEQ ID NO. 12). The B10 α N-terminal PCR primer was 5′-GGAGATCTATGAGCTCTCTGGTACCGCGGGGCTCTAAAGTCTTACAGATCCCAA-GTC-3′ (SEQ ID NO. 13).

The DNA construct encoding the fusion protein containing a hexahistidine tail was cloned into plasmid pPR998; the resultant plasmid was transformed into *E. coli* strain BL21, and cultured as described above in (C). The strain BL21 was selected because it is *Lon*⁻ and *OmpT*⁻, so that fusion proteins expressed in BL21 may be less susceptible to protease degradation. Furthermore, BL21 may be induced with lactose as well as IPTG, because it is *lac*+. The MBP-scTCR$_{HH}$ was first purified utilizing amylose affinity chromatography, followed by nickel affinity chromatography as described below.

Example 2: Isolation, Purification, Denaturation and Refolding of Single Chain T Cell Receptors

A. Development of Nickel Affinity Chromatography to Enhance Folding of MBP D10-scTCR$_{HH}$

MBP D10-scTCR$_{HH}$ was purified under non-reducing conditions utilizing amylose affinity chromatography, as described in Example 1(C) above. In order to separate monomeric MBP D10-scTCR from aggregates and to minimize non-covalent aggregation, a method of nickel affinity chromatography was developed. One hundred mg of amylose purified fusion protein was denatured with a buffer (pH 8.0) containing 6 M GuHCl, 10% glycerol and 0.5 M NaCl (pH 8.0) at room temperature for thirty minutes. The denatured protein was filtered through a 0.2 $\mu$ filter and loaded onto a 20 ml XK 26/20 Ni-NTA column (Qiagen, Studio City, CA) that was equilibrated with binding buffer A containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 10% glycerol, and 6 M GuHCl. The flow rate was 1 ml/min. After washing the column with ten column volumes of binding buffer, a refolding gradient was initiated as recommended by the supplier. A 90 minute linear gradient was formed from 100% binding buffer A to 100% refold buffer B containing 50 mM Tris-HCl (pH 8.0), 20% glycerol, 0.5 M NaCl, and 0.2 M GuHCl at 1.0 ml/min using the Pharmacia FPLC system. The column was washed with an additional four column volumes of buffer B and the bound material eluted in buffer B containing 250 mM imidazole. The flow-through contained most of the *E. coli* contaminating proteins as well as the C-terminally truncated fusion protein. The yield of the eluted fusion protein was typically 25-30%. The purified fusion protein was subjected to SDS-PAGE under both reducing and nonreducing conditions. Under reducing conditions, the fusion protein migrated as a single species

with a molecular weight of about 70 kDa. Under nonreducing conditions the fusion protein migrates as a doublet, each species having a molecular weight of about 70 kDa. Since both species migrate as a singlet under reducing conditions, they seem to represent two distinct disulfide bonded isoforms of the fusion protein. The nickel-affinity chromatography can be scaled up by at least 10-fold by modifying the refolding gradient in an appropriate manner.

B. 3D3 Immunoaffinity Chromatography of Nickel Affinity Chromatography Purified MBP D10-scTCR$_{HH}$

The fusion protein processed by nickel affinity chromatography was applied to an anticlonotypic 3D3 immunoaffinity column. The bound protein was eluted with 50 mM citrate (pH 3.0). The 3D3 immunoaffinity chromatography specifically isolated one isoform (the top band) of at least two, distinct intramolecular sulfhydryl-bonded isoforms (data not shown). Since 3D3 is an anticlonotypic antibody, the eluted material appears to be in native-like conformation. The yield of 3D3-eluted MBP D10-scTCR$_{HH}$ was between 13-22%.

C. Development of Superdex 200 PG SEC for the Isolation of Monomeric Fusion Protein

Because of the presence of minor amounts of covalent aggregation observed after 3D3 immunoaffinity chromatography, a size exclusion chromatography step was developed to isolate monomeric fusion protein from other higher molecular weight aggregates. This step was necessary for subsequent thrombin cleavage experiments, described below.

To avoid losses of purified protein during ultrafiltration, the 3D3 eluted fusion protein was first concentrated using the nickel affinity chromatography

carried out under native conditions, as described above. The column was equilibrated with binding buffer (pH 7.0) containing 0.1 M sodium phosphate and 0.5 M NaCl. The bound material was eluted with 250 mM imidazole. The concentrated fusion protein was then injected onto the Superdex 200 PG XK16/60 Pharmacia FPLC column that was equilibrated with 50 mM Tris-HCl (pH 8.0) and 2 mM $CaCl_2$. The flow rate was 1 ml/min. The chromatogram indicated the separation of aggregate peaks from the monomeric fusion peak, as shown in Figure 7. The fractions under the monomeric peak were pooled. The purity and the integrity of the monomeric fusion protein was monitored using SDS-PAGE under nonreducing conditions (data not shown). The yield after Superdex purification was approximately 30%. Following Superdex purification, the MBP-scTCR can be further purified by utilizing amylose affinity purification, followed by further concentration of the protein utilizing CENTRIPREP/CENTRICON™ (Amicon, Beverly, MA).

D. Digestion of Purified Soluble MBP-scTCR

There may be instances in which it is desirable to obtain the scTCR domain free from the MBP domain. To liberate the scTCR from the fusion protein, the tether can be digested with the highly sequence specific protease thrombin, that recognizes and cleaves at a unique site present in the tether of the fusion protein.

The soluble MBP-scTCR does not precipitate out of solution when dialyzed into a number of different buffers at physiological pH. It can be treated with the enzyme thrombin, which cleaves after four residues into the sequence leu-val-pro-arg-gly-ser (SEQ ID NO. 9). This sequence is present in the tether that connects the MBP domain to the scTCR domain in the fusion protein. Upon

exposure to this protease, the fusion protein is cleaved into its constituents-MBP and scTCR, and some of the scTCR precipitates. However, a significant fraction of the scTCR in the cleaved material remains soluble, as does essentially all of the MBP.

To digest the fusion protein, 18 mg of native-like monomeric fusion protein (0.2 mg/ml) in a buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$ at a concentration of approximately 0.2 mg/ml was digested with 0.5 mg of thrombin at 37°C for 16 hours. SDS-PAGE analysis of the soluble thrombin digested fusion protein indicated that digestion was complete and quantitative, resulting in only two major bands. Thrombin migrated at an apparent molecular weight of about 33 kDa under reducing conditions, whereas under non-reducing conditions it co-migrated with MBP at about 44 kDa. This mobility has allowed the assessment of the removal of thrombin in subsequent purification steps. Approximately 98% of the soluble scTCR was recovered after thrombin digestion.

E. Purification of D10 scTCR following Thrombin Digestion

Eighteen mg of thrombin-digested fusion protein was filtered through a 0.22 micron filter and applied to a 2 ml nickel column equilibrated with 50 mM Tris-HCl (pH 8.0). After binding, the column was washed with ten column volumes of binding buffer (pH 8.0) and eluted with binding buffer containing 250 mM imidazole. Fractions were analyzed for protein content by monitoring A280 and the appropriate fractions pooled. The pooled material was dialyzed into a buffer suitable for further crystallization experiments containing 20 mM MES, 0.02% sodium azide (pH 6.8). The protein sample was further concentrated to 2.5 mg/ml using a CENTRICON 30k MW cutoff. The final yield of the concentrated D10 scTCR protein was approximately 21-

25%. Non-reducing 12% SDS-PAGE analysis demonstrated complete and quantitative cleavage of soluble D10 scTCR (data not shown). There was no evidence of any contaminating uncut fusion protein, MBP, or thrombin.

Example 3: Biophysical and Structural Characterization Studies

Biophysical and structural characterization studies indicated that the purified D10 scTCR exhibited the expected native-like properties.

A. Amino Terminal Sequence Analysis of Purified D10 scTCR

A 10 μg aliquot of purified D10 scTCR was buffer exchanged into methanol using a Prospin column. The D10 scTCR was then subjected to amino-terminal sequence analysis using an Applied Biosystems Model 430A sequencer/120A PTH analyzer. The data showed the sequence to be $NH_2$-GSAVSQSP (SEQ ID NO. 14). This corresponds exactly to the amino acid sequence predicted by the nucleic acid sequence encoded in the plasmid (see Figure 3).

B. Size Exclusion Chromatography (SEC) or Isolated Single Chain TCR

In order to assess whether isolated D10 scTCR was monomeric in solution, the protein was subjected to analytical Superdex 75 size exclusion chromatography (SEC). A 50 μg sample of D10 scTCR in 20 mM MES (pH 6.0) at a concentration of 2 mg/ml was injected onto a Superdex 75 HR 10/30 column equilibrated with 50 mM sodium phosphate, 0.2 M sodium sulfate, 10% glycerol (pH 6.8). The column was run at a flow rate of 0.5 ml.min. Bio-Rad size exclusion standards were also run using identical conditions. Results, shown in Figure 8, indicate that at 2 mg/ml, concentrated isolated D10 scTCR remains monomeric. Solid

line indicates the results with D10 scTCR; dashed line indicates the standards separated under identical conditions. Concentrated D10 scTCR (shown at arrow in Figure 8) appeared at the elution volume predicted from the molecular size of the monomer. As shown in Figure 9, the in-solution calculated native molecular weight generated from SEC analysis was approximately 28,184, which is in good agreement with the molecular weight of 27,907 calculated from the primary amino acid sequence.

C. Electrospray-Mass Spectrometry (ES-MS) Analysis of Purified D10 scTCR

The pure D10 scTCR sample was desalted on reverse-phase HPLC using a Vydac C4 column (4.6 x 250 mm). One hundred micrograms were injected onto a column equilibrated with 0.1% TFA/water and eluted with a 0.1% TFA/95% acetonitrile/5% water gradient. The peak fraction was dried in a speed vac. ES-MS was performed using a VG Biotech Bio-Q instrument with quadruple analyzer (M-Scan Inc., Westchester, PA). Myoglobin was used to calibrate the instrument. Sample aliquots of 10 $\mu$l were injected into the instrument source. Elution was carried out using a 1:1 v/v methanol:water solution containing 1% acetic acid at a flow rate of 4 $\mu$l/min. The sample gave a strong positive ion ES-MS spectrum with a major series of possible multiply-charged ions. When deconvoluted, the data showed a major component with molecular mass of 27889.8 Da, as shown in Figure 10. Other higher molecular mass species in Figure 10 may represent phosphate or sulfate adducts. Figures 11 and 12 show the same analysis on other sample preparations, and illustrates the variable levels of the "adducted" forms. The expected molecular mass calculated from the primary amino acid sequence, including the hexahistidine carboxy-terminus, is 27892.7. The mass spectrometry data were in close agreement with the expected

molecular mass; the observed values are 27,889.8 (Figure 10), 27892.4 (Figure 11), and 27,891.5 (Figure 12), with an average of 27,891.2. The average is within 1.5 daltons of the predicted molecular mass, which is within the range of experimental error for ES-MS analysis.

D. Isoelectric Focusing of Isolated D10 Single Chain TCR under Native Conditions

Isoelectric focusing (IEF) analysis was performed on purified D10 scTCR to assess the isoelectric homogeneity of the material used for crystallization studies. A 35 μg aliquot that was judged to be greater than 95% pure based on silver-stained SDS-PAGE, was analyzed using a Servalyt precoat pI 3-10 gel using a Pharmacia Multiphor flatbed unit. The gel was fixed with 20% TCA for ten minutes and rinsed with MilliQ grade water. The bands were visualized using Serva blue.

A predominant species was present at a pI of 8.8 (data not shown). This value is in agreement with the theoretical value of 8.9 determined from the primary amino acid sequence. Small amounts of minor species were apparent with pI's very close to that of the major species.

E. Circular Dichroism (CD) Spectroscopy of D10 Single Chain TCR

Circular dichroism (CD) spectroscopy can be used to characterize the structural integrity of purified proteins. CD analysis was carried out on D10 scTCR in 20 mM MES (pH 6.8) at a concentration of 0.2 mg/ml. Far-ultraviolet (Far-UV) CD spectra were recorded using a 1-mm path cell on a Model 62 DS CD instrument (Aviv Associates, Lakewood, NJ). Data were collected using a time constant of 1 second at every 0.25 nm, and with a 1-nm constant spectral band width at 25°C. As shown in Figure 13, D10 scTCR appears to be predominantly in the beta pleated sheet form, i.e.,

stabilized predominantly by beta sheet secondary structure, as is expected for a correctly folded recombinant single chain T cell receptor protein that is a member of the immunoglobulin superfamily of proteins.

## Example 4: Single Chain T cell Receptors are Produced in a Biologically Relevant Conformation

### A. Materials

All chemicals, antigens, and adjuvants were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Click's Eagle's high amino acid medium (EHAA; Irvine Scientific, Santa Ana, CA) containing 5% fetal calf serum was used throughout the studies. Hank's balanced salts solution (HBSS) was purchased from Mediatech, Inc. Herndon, VA. Recombinant mouse interleukin-1α (IL-1α) was purchased from R & D Systems, Minneapolis MN, and tritiated thymidine from ICN Radiochemicals, Irvine, CA.

B10.BR mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

The peptide corresponding to the sequence of residues $His^{134}$-$Gly^{146}$ in hen egg conalbumin was purchased as a custom synthesis from Coast Scientific, San Diego, CA. The sequence of this synthetic peptide, referred to herein as "pwt", is HRGAIEWEGIESG (SEQ ID NO. 15).

The D10.G4.1 AKR mouse T cell clone (TIB 224), and the CH-1 mouse B cell lymphoma (TIB 221) were obtained from ATCC, Rockville, MD.

The I-$A^k$-specific mAb was produced as an ascites from the hybridoma cell line 11-5.2.1.9 (TIB 94) obtained from ATCC, Rockville, MD. The mAb specific for a clonotypic determinant of the D10 TCR was produced as a culture supernatant of the 3D3 hybridoma described by Kaye, J., *et al.* (*J. Exp. Med.* 158:836-856 (1983)).

B. D10 MBP-scTCR Fusion Protein Shares a Clonotypic Determinant with the D10 Cell

Because immunochemical techniques demonstrated that a significant proportion of the fusion protein preparations possessed correctly folded variable regions as demonstrated by their reactivity with anti-clonotypic 3D3 antibody bound to a membrane, *in vitro* experiments were designed to investigate whether the scTCR variable region of the fusion protein could compete with the T cell surface TCR for the antibody. These experiments investigate whether MBP-scTCR binds the 3D3 mAb in solution, and whether there is a serological similarity between paired variable regions in the fusion protein and the native cell surface form of the D10 TCR.

The D10 T cell clone can be triggered to proliferate by 3D3 antibody in the presence of IL-1. From antibody titration experiments a 1:4,000 dilution of 3D3 hybridoma cell culture supernatant was chosen as a limiting quantity of mAb for the stimulation of D10 T cell proliferation. 50 $\mu$l volumes of this dilution of supernatant were incubated with varying amounts of D10 MBP-scTCR or B10 MBP-scTCR for one hour at room temperature prior to the addition of 2 x $10^4$ D10 T cells and recombinant IL-1 (20 units/ml) and further incubation for 72 hours at 37°C. The cultures were set up in triplicate in 200 $\mu$l volumes in 96-well round-bottomed plates. D10 T cell proliferation was assessed by pulsing each culture with 1 $\mu$Ci $^3$H-thymidine for the final 12-16 hours of incubation. The cells were harvested on a Tomtec harvester (Orange, CT) and radioisotope incorporation measured using a beta-plate scintillation counter (Wallac, Gaithersburg, MD). The data are presented in Figure 14. The solid bar indicates the D10 proliferative response induced by the 1:4,000 dilution of mAb 3D3 and recombinant interleukin-1 (IL-1) (20

units/ml), and the broken bar, the background response with IL-1 alone. Solid symbols indicate the D10 proliferative response with antibody and IL-1 in the presence of the concentrations of MBP D10-scTCR indicated on the abscissa. Open symbols indicate the response with MBP B10-scTCR.

The D10 MBP-scTCR competed specifically with the D10 cell surface TCR whereas the unrelated B10 MBP-scTCR was completely without effect. The sigmoidal titration curve of D10 MBP-scTCR competition (solid symbols in Figure 14) allows the specific activity of a fusion protein preparation to be defined as the protein concentration yielding 50% inhibition of the maximal response to a standard amount of 3D3 mAb. The assay can be used in fusion protein refolding experiments to monitor the successful reconstitution of the clonotypic, conformational determinant recognized by the 3D3 antibody.

C. The D10 scTCR can Compete with the D10 Cell Surface TCR for Antigen

The antigen ligand of the D10 TCR is a peptide fragment of hen egg conalbumin bound to the mouse MHC-I-$A^k$ class II molecule (Nakagawa, T.Y., *et al.*, *Eur. J. Immunol.* *21*:2851-2855 (1991)). The ligand was formed on the surface of the I-$A^k$-expressing CH-1 B cell lymphoma cells by incubating the cells in Click's medium at a concentration of 5 x $10^7$/ml with the pwt synthetic peptide (SEQ ID NO. 15) at a concentration of 100 µg/ml for 2 hours at 37°C. The peptide treated cells were washed three times in HBSS and fixed by a 30 second exposure to a 0.05% solution of glutaraldehyde (v/v in HBSS) at a cell concentration of 5 x $10^6$/ml. The fixation reaction was terminated by adding Click's medium, and the cells were washed three times prior to use as antigen presenting cells in a D10 T cell proliferation assay.

The T cell proliferation assay was performed in 96-well round-bottomed plates in 200 μl cultures containing 1 x $10^4$ D10 cells and varying numbers of peptide-treated, fixed CH-1 cells. Control cultures were set up in which the background proliferation of D10 cells was measured in the presence of equivalent numbers of fixed CH-1 cells that had not been preincubated with the pwt peptide (SEQ ID NO. 15). A purified preparation of D10 scTCR was added to peptide-stimulated cultures in order to investigate the ability of the soluble receptor to bind to the ligand complex of I-$A^k$ and peptide, and thereby block D10 T cell activation. As a positive control, the I-$A^k$-specific mAb 11-5.2.19 was added to some cultures. D10 cell proliferation was assessed by a $^3$H-thymidine pulse for the final 16 hours of incubation. Harvesting and scintillation counting were performed as described above for the 3D3 antibody stimulated D10 T cell proliferation assay. Isotope incorporation in the control cultures was subtracted from that in the antigen stimulated cultures to calculate Δ cpm values representing the antigen-specific proliferative response.

The data from the Experiment are shown in Figure 15 (circles, D10 T cell response without any addition; diamonds, D10 T cell response in the presence of 4 μM D10 scTCR; squares, D10 T cell response in the presence of 8 μM D10 scTCR; triangles, D10 T cell response in the presence of 1:100 dilution of the I-$A^k$-specific mAb 11.5.2.19.) The CH-1 cells were titrated between 1000 and 50 cells per culture, and it is apparent that within this range the T cell response was limited by the amount of antigen. As expected, the D10 response could be virtually completely inhibited by the addition of ascites containing the I-$A^k$-specific 11-5.2.19 mAb (triangles). When added at 4 (diamonds) and 8 (squares) μM, inhibition of the response

was observed with a highly purified preparation of the soluble D10 scTCR. There was approximately 50% inhibition at the higher concentration. Previously published data indicate that the affinity of the TCR for the antigenic complex of peptide and MHC class II molecules is low compared with that of antibody-antigen interactions. By two different methods the dissociation constant for the TCR-ligand interaction is estimated to be approximately 1 x $10^{-5}$ to 6 x $10^{-5}$ M: that is orders of magnitude weaker than comparable antigen-antibody interactions (Matsui, K., *et al.*, *Science* 254:1788-1791 (1991); Weber, S., *et al.*, *Nature* 356:793-795 (1992)). It is not surprising, therefore, that the soluble TCR preparation competes with the cell surface D10 TCR much less efficiently than the anti-I-$A^k$ antibody does. The observed ability of the soluble D10 scTCR to compete indicates that it binds the ligand formed by the pwt conalbumin peptide (SEQ ID NO. 15) and I-$A^k$ molecule. This is evidence that the antigen binding site of the recombinant protein quite faithfully emulates that of the native T cell surface protein.

D. Characterization of D10 scTCR using mAbs Specific for α or β Chains

Purified D10 scTCR was characterized by Enhanced Chemiluminescence (ECL) Amersham slot-blot analysis (Amersham, Arlington Heights, IL) using the mAb specific for Vα2 (Pharmingen, CA) and Vβ8 (KJ16; Haskins, K.J., *et al.*, *J. Exp. Med.* 160:452-71 (1984)) TCR segments. The purified scTCR reacted well with these V region family specific antibodies, as well as with the clone-specific antibody 3D3 (data not shown). The Vα2-specific antibody is also useful in western blotting under non-reducing conditions. The antibody does not recognize the Vα2 epitope if the D10 scTCR has been reduced, suggesting that

conformation of the epitope depends upon the two framework cysteines in the Vα2 domain being in close proximity to each other (i.e., covalently linked by a sulfhydryl bond). Thus, reaction of the Vα2 mAb with the scTCR supports the contention that the sulfhydryl bonds of D10 scTCR are correctly formed.

E. Reactivity of MBP B10-scTCR$_{HH}$ with Conformation-Sensitive Monoclonal Antibodies

An anti-clonotypic mAb, 8G2, as well as a Vα11-specific antibody, 1F2, were used to study the conformation of the MBP B10-scTCR$_{HH}$ fusion protein. Fusion protein was purified by amylose affinity chromatography under non-reducing conditions, and nickel affinity chromatography under reducing and denaturing conditions. After dilution to 100 μg/ml with 6 M GuHCl, 10 mM Tris-HCl (pH 8.0) and dialysis against PBS (pH 7.4), the purified protein was applied to an immunodyne activated membrane (Pall, Inc.) using a BioRad slot blot apparatus. After blocking with 10% non-fat dry milk for one hour, the membranes were incubated with either 1F2 or 8G2 monoclonal antibodies at 2 μg/ml for 12 hours at room temperature. Following extensive washing, the membranes were incubated with HRP conjugated goat anti-mouse IgG (1:5000 dilution) for 1 hour. The membranes were washed extensively and were then developed with Amersham ECL developer. Although 1F2 mAb reacted with amylose- and nickel-affinity purified B10 fusion protein, only amylose-affinity purified fusion protein reacted with 8G2 mAb. Since nickel purification was performed under reducing and denaturing conditions, it is plausible that the conformation required for reactivity to 8G2 was not achieved. The reactivity of 1F2 suggests, however, that at least the α-chain of the dialyzed fusion protein is folded in a native-like conformation.

F. Conformation Sensitive Immunoassays

The following immunological assay was used to assess the degree of reactivity of the recombinant TCR proteins with the appropriate conformation specific anti-clonotypic antibodies. Up to 100 ng of purified protein was covalently bound, via epsilon amino groups of lysine residues, to a chemically activated hydrophilic PVDF membrane (Immobilon AV; Amersham, Arlington Heights, IL). This was achieved by vacuum filtration of the antigen using a dot blot apparatus, followed by incubation of the membrane at room temperature overnight between two sheets of blotting paper prewetted in PBS. The proteins on the membrane were then either: (i) left in the native state, (ii) denatured by boiling, or (iii) both denatured and reduced by boiling in the presence of 2-mercaptoethanol (2 Me). Unreactive sites were then capped by incubation of the membrane in a solution containing 1 M $NaHCO_3$ and 10% monoethanolamine. A panel of conformation sensitive monoclonal antibodies was then used for probing the immobilized recombinant proteins. After washing in buffers containing 0.2% Tween 20, the membrane was incubated with a horseradish peroxidase coupled secondary antibody. The signal was finally detected on X-ray film by employing the ECL chemiluminescence detection system (Amersham).

D10 and B10 scTCRs were digested with thrombin or left undigested, and then blotted onto duplicate membranes. One membrane was exposed to native conditions, while the other was exposed to the denaturing conditions described above. The membranes were then probed with a panel of monoclonal antibodies including 3D3, 1F2, RR8, and 8G2. The filters were then processed and further developed as described in section E, above. The antibodies were extremely conformation sensitive, as they were unable to react with the denatured sample.

A conformation specific assay utilizing the D10 clonotype-specific antibody, 3D3, was performed on two samples of the D10 scTCR. One possessed the wild type sequence of the variable regions, whereas the other had a deletion of 15 amino acids at the carboxy terminus of the J region. The loss of the J region sequence obliterated the positive signal obtained with the complete variable region that included the entire sequence of the J region. Independent results indicated that certain residues in the J α region are important for reactivity with the MAb 3D3 when this TCR is expressed on the surface of cultured eucaryotic cells, further corroborating the results shown here.

The Vα11 specific antibody, 1F2, reacts with the native (heat-sensitive) epitope present on the α chain of the MBP B10-scTCR fusion protein encoded by the plasmid 23/528. However, the recombinant protein is not reactive with the anti-clonotypic antibody, 8G2, indicating that the conformation of this protein is not identical to that of the TCR appearing on the surface of B10 cells. In contrast, anti-clonotype reactivity can be observed for this scTCR if the linker connecting the β and α chains is altered. This linker (the 3XG/FLAGG linker) has an additional eight amino acids (DYKDDDDK (SEQ ID NO. 7), the FLAGG sequence) inserted proximal to the carboxy terminus of the β chain. This new protein, encoded by the plasmid 6/538, is essentially indistinguishable from the TCR appearing on the surface of B10 cells as judged by reactivity to both 8G2 and 1F2. Both of these antibodies recognize epitopes that are lost upon denaturation by boiling and thus they are considered to be exquisitely sensitive indicators of TCR conformation.

Example 5: Recombinant scTCR can Immunize Syngeneic Mice to Produce a Specific Antibody Response

A. Materials

Materials described previously are not mentioned again here. Dulbecco's phosphate buffered saline (PBS) was purchased from Biowhittaker, Walkersville, MD. Staining buffer for immunofluorescence was prepared by supplementing PBS with 5% fetal calf serum (FCS) and 0.1% sodium azide. Mouse T cell enrichment columns were purchased from R & D Systems, Minneapolis, MN.

AKR mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin antibody was purchased from Cappel, Durham, NC. The Vβ-8- and Vα-11-specific FITC-conjugated monoclonal antibodies were purchased from Pharmingen, San Diego, CA.

The B10 T cell clone was a gift from Stephen Hedrick and Gerald Siu (University of California, San Diego, CA)

The following soluble TCRs were produced, utilizing the baculovirus expression system, as chimeric proteins in which the α and β chains of the TCR are linked to the Fc portion of mouse IgG1 to form a disulfide-bonded dimeric molecule. The T cell clones from which these chimeric TCRs were made were B10, D10, and two clones derived from the NOD mouse. The NOD clones BDC 2.5 and BDC 6.9 are pancreatic islet antigen specific and restricted by $I\text{-}A^{g7}$ (K. Haskins, Barbara Davis Center for Childhood Diabetes, Denver, CO).

B. The D10 scTCR Stimulates an Antibody Response in the AKR Mouse

Potential applications of scTCR include the production of antibodies, and the modulation of immune responses via

the induction of an immune response that targets T cells bearing a TCR that shares antigenic epitopes with the scTCR. For these to be feasible the scTCR must be immunogenic: that is, it has to be able to induce an immune response in a naive animal. The successful production of TCR-specific antibodies in numerous laboratories clearly demonstrates that the D10 TCR (Kaye, J., *et al, J. Exp. Med.* 158:836-856 (1983)) and other TCR (for example: Sitkovsky, M.V., *et al., J. Immunol.* 129:1372-1376 (1982); Samelson, L.E., *et al., Proc. Natl. Acad. Sci. USA* 80:6972-6973 (1983); Infante, A.J., *et al., Current Protocols in Immunology* 1:(1982)) are immunogenic. Previously, the immunizations were mainly done with intact T cells injected into either mice of a different strain or animals of a different species. Neither the question of immunogenicity of the TCR protein alone, nor that of TCR immunogenicity in a syngeneic animal was addressed. Previous data suggested that the injection of the viable D10 T cell clone could stimulate an antibody response by a direct interaction of the clone with B cells expressing cell surface immunoglobulin molecules with specificity for D10 TCR epitopes (Tite, J.P., *et al., Exp. Med.* 163:189-202 (1986)). There was some evidence that immune recognition in this situation involved only the binding of TCR to specific immunoglobulin. The observed antibody responses need not necessarily have involved antigen-processing of the TCR by APC, and the presentation of TCR-peptide epitopes to $CD4^+$ T-helper cells in a MHC class II restricted fashion. It was not previously obvious, therefore, that recombinant soluble TCR would be immunogenic, because just like any other protein antigen, in order to stimulate antibody formation, the TCR would have to be processed to produce peptide-class II molecular

complexes with the ability to specifically activate T-helper cells.

In humans, for some types of TCR vaccination strategy to succeed, the TCR of interest would have to able to induce an immune response in an individual of identical genotype to that from which the TCR was derived. Since the genes encoding the variable region of the TCR are formed by unique somatic rearrangements of germline gene segments in each developing T cell, every TCR potentially possesses clonotypic epitopes that were not available in sufficient amounts during ontogeny of the immune system to induce tolerance. Consequently, if a recombinant soluble TCR is immunogenic in a syngeneic animal, the immune response might be expected to be directed towards clonotypic epitopes of that TCR rather than to conserved regions that are shared with other TCR.

The D10 T cell clone was originally derived from the draining lymph nodes of immunized AKR mice (Kaye J., *et al., J. Exp. Med. 158*:836-856 (1983)). To investigate the immunogenicity of the D10 scTCR in a syngeneic situation, it was therefore injected into AKR mice. 6-8 week old AKR mice were immunized subcutaneously in the hind limbs with 25 μg of purified D10 scTCR emulsified in complete Freunds' adjuvant. After 1 month the mice were boosted by intraperitoneal injection of an additional 20 μg of scTCR in PBS, and 4 days later the mice were bled from the retro-orbital plexus. Sera were collected, heat inactivated at 56°C for 30 minutes, diluted in staining buffer, and stored at 4°C. The analysis of the specificity of one antiserum for cell surface TCR is shown in Figure 16. 1 x $10^6$ D10 clone, B10 clone or normal AKR mouse splenic T cells were incubated at 4°C for 30 minutes with the indicated dilutions of the antiserum in staining buffer in 96-well round bottomed plates. The AKR mouse T cells were purified

by applying a whole spleen cell population to T cell columns according to the manufacturers instructions (R & D Systems, Minneapolis, MN). After incubation with antiserum the cells were washed with staining buffer and incubated under the previous conditions with FITC-conjugated goat anti-mouse immunoglobulin antibody at a 1:200 dilution. After washing to remove unbound antibody, cell-surface fluorescence intensity was measured in the FACScan. From the third row of histograms in Figure 16, it can be seen that a 1:10 dilution of the antiserum stained the D10 cells, but not the B10 or AKR T cells. The data indicate that the AKR antiserum contains antibody specific for an epitope unique to the D10 cell surface form of the TCR. Direct immunofluorescence with FITC conjugated monoclonal antibodies specific for TCR V$\beta$-8 and V$\alpha$-11 indicated that the D10, B10 and AKR T cells expressed readily detectable cell-surface TCR at the time the experiment was performed (Figure 16, second row).

It has previously been demonstrated that D10 T cells are uniquely sensitive to activation via antibodies specific for their TCR (Tite, J.P., *et al.*, *Exp. Med.* *163*:189-202 (1986)). Most antibodies specific for the TCR of other T cell clones inhibit their function *in vitro* (for example, Haskins, K., *et al.*, *Exp. Med.* *160*:452 (1984)). The anti-D10 scTCR antiserum was tested for its ability to stimulate D10 T cell proliferation. $4 \times 10^4$ D10 T cells and $5 \times 10^5$ X-irradiated AKR spleen cells were incubated in 200 $\mu$l Click's medium in 96-well round-bottomed plates with or without the addition of a 1:10 dilution of the AKR antiserum. This was the antiserum analyzed by cell-surface immunofluorescence in the experiment described above. After 48 hours incubation at 37°C, T cell proliferation was measured by pulsing with $^3$H-thymidine, harvesting, and

counting in a liquid scintillation counter as previously described. The results are shown in Figure 17.

The data clearly indicate that the antiserum contained antibodies capable of stimulating a vigorous proliferative response (Figure 17), and therefore support the conclusion that immunization of the AKR mouse with D10 scTCR stimulated the production of antibodies to the receptor.

The ability of the serum antibodies to bind the D10 cell-surface form of the TCR further indicates that the scTCR presents epitopes to the immune system that are identical to those of the native molecule. The biological data therefore provide further evidence that the conformation or folding of the recombinant protein is similar to that of the TCR produced by the D10 T cell.

C. Specificity of Antiserum to D10 scTCR

Antisera to both BDC 2.5-IgG1 and to D10 scTCR, diluted 1:5000, were analyzed by sandwich ELISA. Soluble TCRs (B10-IgG1, D10-IgG1, BDC 2.5-IgG1 and BDC 6.9-IgG1) were adsorbed to the 96-well plates at 4 μg/ml concentration. Ninety-six well Maxisorp Immunomodules (Nunc, Naperville, IL) were incubated overnight at 4°C with 100 μl per well of soluble TCR in borate saline buffer at a concentration of 4 μg/ml. Plates were washed 6 times with wash buffer (borate saline buffer pH 8.3 with 0.05% Tween 20) after this and between each subsequent step. Plates were blocked for 1 hour at room temperature with 1% BSA in borate saline buffer. Next, serially diluted antiserum samples in 1% BSA were added in duplicate. Following standard protocols, after an overnight incubation at 4°C, alkaline phosphatase (AP)-labeled goat anti-mouse (kappa + lambda light chain specific) (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as a detecting antibody, diluted 1:500, in 1% BSA overnight at 4°C. Plates were developed with Sigma 104 phosphatase substrate

(Sigma, St. Louis, MO) in diethanolamine. Absorbance was measured at 405 nM. Results are shown in Figure 18.

D. Generation of a Monoclonal Antibody to D10 scTCR

The D10 scTCR was used to generate a monoclonal antibody. Briefly, Balb/c mice were injected subcutaneously with 30 μg D10 scTCR in complete Freund's adjuvant. After 14 days the mice were boosted by intraperitoneal injection of 20 μg D10 scTCR in PBS, and 14 days later the mice were again boosted by intraperitoneal injection of an additional 20 μg D10 scTCR in PBS. Five days later the mice were sacrificed, and spleen cells were used to generate hybridomas. The hybridomas producing antibodies were selected. One hybridoma produced the monoclonal antibody 3E9G2.

Experiments were conducted demonstrating the ability of the monoclonal antibody 3E9G2 to stimulate D10 T cell proliferation. The assay was performed as described above, except that the antibodies 3E9G2 and 3D3 (positive control) were titrated from 1/10 down to $1/10^6$. Background wells contained $4 \times 10^4$ D10 cells, and $5 \times 10^5$ X-irradiated spleen cells only. The results, shown in Figure 19, indicate that the monoclonal antibody 3E9G2 is capable of stimulating a vigorous proliferative response.

FACS analysis was performed as described above using the monoclonal antibody 3E9G2 instead of serum. Results indicated that the 3E9G2 antibody is clonotype-specific to D10 scTCR: 3E9G2 bound to D10 T cells, but not to purified AKR splenic T cells or control ascites (data not shown).

E. Surface Plasmon Resonance (SPR) Binding Experiments Demonstrate the Specificity of 3E9G2

The binding of mAb 3E9G2 to immobilized D10 scTCR was studied using a BIAcore™ biosensor (Pharmacia LKB

Biotechnology Inc., NJ) technique. The instrument can detect binding of soluble analytes to a ligand immobilized on a dextran-coated chip in real time (Johnsson *et al., Anal. Biochem. 198*:268-277 (1991), Malmqvist, *Current Opinion in Immunology 5*:282-286 (1993)). For binding experiments, pure D10 scTCR proteins were coupled to dextran surface by standard amine coupling chemistry (O'Shannessy, *et al., Anal. Biochem. 205*:132-136 (1991)). Throughout the binding experiments, a flow of HBS (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) at 5 μl/min was maintained. For binding and epitope mapping experiments, twenty μl each of mAbs 3E9G2, 3E9G12, 3D3, Vα2 and Vβ8 were injected over immobilized surfaces at a flow rate of 5μl/min. 3E9G12 is a control antibody. At the end of each binding cycle, the biosensor surface was regenerated with 10 mM HCl. All binding experiments were conducted at 25°C. The SPR signal was recorded as a resonance unit (RU) *versus* time and was plotted as a "sensogram", as shown in Figures 20-24.

SPR analyses confirmed that mAb 3E9G2 bound tightly to immobilized D10 TCR proteins; control antibody 3E9G12 under identical conditions did not bind (Figure 20). Moreover, mAb 3E9G2 binding to immobilized D10 TCR is blocked by mAbs Vα2 (Figure 21) and 3D3 (Figure 22), implying that the binding site for 3E9G2 overlaps binding sites for 3D3 and Vα2. In contrast, mAbs Vβ8 and 3E9G2 have distinct binding sites on D10 TCR, since Vβ8 does not block binding of 3E9G2 (Figure 23) and 3E9G2 does not block Vβ8 binding (Figure 24). Conformational sensitive slot-blot and FACS analyses described above thus confirmed that 3E9G2 is a conformational-sensitive D10 clone-specific mAb.

Example 6: Vaccination with the B10 MBP-scTCR Fusion Protein Modulates the T Cell Response to Pigeon Cytochrome C

A. Materials

Materials described previously are not mentioned again here. B10.A and B10.BR mice expressing I-$A^k$ were purchased from The Jackson Laboratory, Bar Harbor, ME.

The Vβ-3-specific FITC-conjugated mAb were purchased from Pharmingen, San Diego, CA.

B. Suppression of T cells Expressing Vα by *in vivo* Treatment with B10 MBP-scTCR Fusion Protein

Mice have been immunized with soluble B10 MBP-scTCR in order to determine whether an immune response against the TCR portion of the recombinant protein can specifically inhibit the *in vivo* priming of T cells bearing TCR which share structural elements with it. Experiments of this type apply what is called herein the 'vaccination approach' to TCR-targeted immunoregulation. The effect of vaccination with the MBP B10-scTCR fusion protein on the response of B10.A mice to cytochrome C has been investigated. The B10 scTCR is composed of Vα-11 and Vβ-16 segments, and the B10.A mouse T cell response, which is directed almost entirely to the carboxy-terminal peptide of cytochrome C complexed with the I-$E^k$ MHC class II molecule, involves an appreciable proportion of $CD4^+$ T cells expressing TCRs with Vα-11 paired to Vβ-3. In this system the immune response to epitopes within the Vα-11 segment might be expected to inhibit the cytochrome C-response of T cells expressing Vα-11. The fusion protein used for vaccination was not intentionally refolded, and therefore it was anticipated that the fusion protein would immunize the mice against linear TCR epitopes.

MHC-I-$E^k$ expressing mice were immunized by intraperitoneal injection with either the MBP B10-scTCR or D10 MBP-scTCR non-native fusion proteins (50-100 μg/mouse) emulsified in complete Freund's adjuvant. A group of control mice were injected with an equivalent amount (100 μl) of complete Freund's adjuvant alone. After approximately one month, MBP-scTCR and control immunized mice were challenged subcutaneously in the hind limbs with 100 μg of pigeon cytochrome C in complete Freund's adjuvant. After a further 7-10 days, the popliteal lymph nodes were removed from these mice and cultured *in vitro* in order to raise short-term T cell lines against cytochrome C using well established procedures (Fitch, F.W., *et al.*, *Current Protocols in Immunology* 1 (1991)). After 5-7 cycles of antigen stimulation and IL-2 expansion, stable cell lines were analyzed for cell surface expression of TCR Vα-11 and Vβ-3, and also for antigen specificity. The Table represents a summary of the data concerning the expression of Vα-11 as determined by direct immunofluorescence in the FACScan using the FITC-anti-Vα-11 mAb.

Table: Vα-11 expression in pigeon cytochrome C stimulated T cell lines derived from I-$E^k$-expressing mice

| TCR immunization | No. Lines tested | % of T cells expressing Vα-11 (mean ± SE) |
|---|---|---|
| MBP B10-scTCR | 7 | 10.5 ± 6.2 |
| MBP D10-scTCR | 5 | 47.5 ± 15.8 |
| Complete Freund's adjuvant control | 8 | 26.5 ± 5.2 |

Compared with the D10 MBP-scTCR vaccinated and complete Freund's adjuvant immunized mice, immunization with the B10 MBP-scTCR fusion protein significantly suppressed the

response of T cells expressing TCR Vα-11. While all cell lines developed a similar CD4-positive/αβTCR-positive phenotype, it appears that following vaccination against the B10 fusion protein, T cells expressing Vα-11 were suppressed, resulting in their reduced frequency in the T cell lines established *in vitro*.

The T cell lines were also analyzed for cytochrome C specificity in the T cell proliferation assay, and for co-expression of TCR Vα-11 and Vβ-3 by two-color immunofluorescence in the FACScan (Figure 25, Figure 26). There was a striking effect of B10 MBP-scTCR immunization on the development of Vα-11/Vβ-3 bearing cells. Of the seven stable cell lines generated from B10 MBP-scTCR immunized animals, only 0512.2 and 0512.4 showed any significant level of TCR Vα-11 or TCR Vα-11/Vβ-3 expression. Figure 19 shows representative data on these two lines along with another, 0512.3, which expressed no TCR Vα-11 or Vβ-3. These findings contrast considerably with the staining patterns seen in cell lines from complete Freund's adjuvant control (Figure 26), and D10 MBP-scTCR immunized animals. Eight out of eight control lines, and four out of five D10 MBP-scTCR immunized cell lines, expressed significant Vα-11. In most instances Vα-11 was co-expressed with Vβ-3. Representative immunofluorescence data for 4 control lines are presented in Figure 26. To date, approximately half of the cell lines generated from TCR immunized mice have been tested for their specificity to cytochrome C. Data shown in Figures 25 and 26 allow the proliferative responses to be compared with the levels of Vα-11 and Vβ-3 expression. There was no apparent specificity of T cell lines from B10 MBP-scTCR vaccinated mice for cytochrome C. The proliferative responses to that antigen were either inappreciable (line 0512.2) or comparable to those in unstimulated cultures (lines 0512.3 and 0512.4). The lack of antigen specificity in these

lines correlated well with the very low levels of Vα-11/Vβ-3 expression. There was no apparent specificity of T cell lines from MBP-B10 scTCR vaccinated mice for cytochrome C. The proliferative responses to that antigen were either inappreciable (line 0512.2) or comparable to that in unstimulated cultures (lines 0512.3 and 0512.4). The lack of antigen specificity in these lines correlated well with the very low levels of Vα-11/Vβ-3 expression. All of the cell lines derived from control mice injected with complete Freund's adjuvant alone have shown specific proliferative responses to cytochrome C. This is indicated by a comparison of the data of Figures 25 and 26, and it can be seen that the ability to respond specifically to antigen correlated well with the Vα-11/vβ-3 phenotype of these T cell lines. It seems apparent from the phenotypic analyses of T cell lines over the short-term that *in vivo* vaccination with B10 MBP-scTCR has a dramatic effect on T cells which bear the Vα11 TCR.

One interpretation of the data is that *in vivo* immunization against Vα-11 epitopes derived from the TCR portion of the fusion protein results in a suppression of pigeon cytochrome C-specific T cells utilizing the Vα-11 gene segment. The data imply that vaccination of humans with soluble TCR containing a V segment utilized by T cells mediating pathological effects--for example, autoimmune tissue destruction--could potentially reduce the clonal frequency of such T cells in the peripheral immune system *in vivo*, and thereby reduce the severity of the pathology.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Soluble Single Chain T Cell Receptors

(iii) NUMBER OF SEQUENCES: 13

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(D) STATE: Massachusetts
(E) COUNTRY: US
(F) ZIP: 02173

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/329,310
(B) FILING DATE: 26-OCT-94
(C) CLASSIFICATION:

PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/347,893
(B) FILING DATE: 01-DEC-94
(C) CLASSIFICATION:

PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/468,131
(B) FILING DATE: 06-JUN-95
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: PCT/US95/13770
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(C) CLASSIFICATION:

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(C) REFERENCE/DOCKET NUMBER: PRO93-07A3 PCT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1187

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG      48
Met Lys Ile Lys Thr Cys Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
 1               5                  10                  15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA      96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
            20                  25                  30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA     144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
        35                  40                  45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG     192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
    50                  55                  60

CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC     240
His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
65                  70                  75                  80

GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC     288
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                85                  90                  95

GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG     336
Ala Gln Ser Gly Leu Leu Ala Gln Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG     384
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC     432
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG     480
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC     528
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175
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CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT      576
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190

TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC      624
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG      672
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA      720
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG      768
Ala Ala Phe Lys Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA      816
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG      864
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG      912
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT      960
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG     1008
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA     1056
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC     1104
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT     1152
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

GAA GCC CTG AAA GAC GCG CAG ACT AAT TCG AGC TC                      1187
Glu Ala Leu Lys Asp Ala Gln Thr Asn Ser Ser
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 395 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ile Lys Thr Cys Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
1 5 10 15

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
20 25 30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
35 40 45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
50 55 60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
65 70 75 80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
85 90 95

Ala Gln Ser Gly Leu Leu Ala Gln Ile Thr Pro Asp Lys Ala Phe Gln
100 105 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
115 120 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
130 135 140

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145 150 155 160

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
165 170 175

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
180 185 190

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
195 200 205

Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
210 215 220

Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225 230 235 240

Ala Ala Phe Lys Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
245 250 255

```
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

Glu Ala Leu Lys Asp Ala Gln Thr Asn Ser Ser
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 796 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..790

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
G AGC TCT CTG GTA CCG CGG GGC TCT GCA GTC TCC CAA AGC CCA AGA          46
  Ser Ser Leu Val Pro Arg Gly Ser Ala Val Ser Gln Ser Pro Arg
                  400                 405                 410

AAC AAG GTG GCA GTA ACA GGA GGA AAG GTG ACA TTG AGC TGT AAT CAG        94
Asn Lys Val Ala Val Thr Gly Gly Lys Val Thr Leu Ser Cys Asn Gln
                415                 420                 425

ACT AAT AAC CAC AAC AAC ATG TAC TGG TAT CGG CAG GAC ACG GGG CAT       142
Thr Asn Asn His Asn Asn Met Tyr Trp Tyr Arg Gln Asp Thr Gly His
            430                 435                 440
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GGG CTG AGG CTG ATC CAT TAT TCA TAT GGT GCT GGC AGC ACT GAG AAA          190
Gly Leu Arg Leu Ile His Tyr Ser Tyr Gly Ala Gly Ser Thr Glu Lys
        445                 450                 455

GGA GAT ATC CCT GAT GGA TAC AAG GCC TCC AGA CCA AGC CAA GAG AAC          238
Gly Asp Ile Pro Asp Gly Tyr Lys Ala Ser Arg Pro Ser Gln Glu Asn
    460                 465                 470


TTC TCC CTC ATT CTG GAG TTG GCT ACC CCC TCT CAG ACA TCA GTG TAC          286
Phe Ser Leu Ile Leu Glu Leu Ala Thr Pro Ser Gln Thr Ser Val Tyr
475                 480                 485                 490

TTC TGT GCC AGC GGG GGA CAG GGG CGG GCT GAG CAG TTC TTC GGA CCA          334
Phe Cys Ala Ser Gly Gly Gln Gly Arg Ala Glu Gln Phe Phe Gly Pro
                495                 500                 505

GGG ACA CGA CTC ACC GTC CTA GGA TCC GAC TAC AAG GAC GAC GAT GAC          382
Gly Thr Arg Leu Thr Val Leu Gly Ser Asp Tyr Lys Asp Asp Asp Asp
            510                 515                 520

AAG AGA TCC GGT GGT GGT GGT TCC GGA GGT GGT GGT TCT GGT GGT TCT          430
Lys Arg Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Ser
        525                 530                 535

GGC GCC CAG CAG CAA GTG AGA CAA AGT CCC CAA TCT CTG ACA GTC TGG          478
Gly Ala Gln Gln Gln Val Arg Gln Ser Pro Gln Ser Leu Thr Val Trp
    540                 545                 550

GAA GGA GAG ACC ACA ATT CTG AAC TGC AGT TAT GAG GAC AGC ACT TTT          526
Glu Gly Glu Thr Thr Ile Leu Asn Cys Ser Tyr Glu Asp Ser Thr Phe
555                 560                 565                 570

GAC TAC TTC CCA TGG TAC CGG CAG TTC CCT GGG AAA AGC CCT GCA CTC          574
Asp Tyr Phe Pro Trp Tyr Arg Gln Phe Pro Gly Lys Ser Pro Ala Leu
                575                 580                 585

CTG ATA GCC ATA AGT TTG GTG TCC AAT AAA AAG GAA GAT GGA CGA TTC          622
Leu Ile Ala Ile Ser Leu Val Ser Asn Lys Lys Glu Asp Gly Arg Phe
            590                 595                 600

ACA ATC TTC TTC AAT AAA AGG GAG AAA AAG CTC TCC TTG CAC ATC ACA          670
Thr Ile Phe Phe Asn Lys Arg Glu Lys Lys Leu Ser Leu His Ile Thr
        605                 610                 615

GAC TCT CAG CCT GGA GAC TCA GCC ACC TAC TTC TGT GCA GCA ACA GGT          718
Asp Ser Gln Pro Gly Asp Ser Ala Thr Tyr Phe Cys Ala Ala Thr Gly
    620                 625                 630

AGC TTC AAT AAG TTG ACC TTT GGA GCA GGG ACC AGA CTG GCT GTG TCC          766
Ser Phe Asn Lys Leu Thr Phe Gly Ala Gly Thr Arg Leu Ala Val Ser
635                 640                 645                 650

CCA TAT CAC CAT CAC CAT CAC CAT TAATGA                                   796
Pro Tyr His His His His His His
                655
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Ser Ser Leu Val Pro Arg Gly Ser Ala Val Ser Gln Ser Pro Arg Asn
 1               5                  10                  15

Lys Val Ala Val Thr Gly Gly Lys Val Thr Leu Ser Cys Asn Gln Thr
            20                  25                  30

Asn Asn His Asn Asn Met Tyr Trp Tyr Arg Gln Asp Thr Gly His Gly
        35                  40                  45

Leu Arg Leu Ile His Tyr Ser Tyr Gly Ala Gly Ser Thr Glu Lys Gly
    50                  55                  60

Asp Ile Pro Asp Gly Tyr Lys Ala Ser Arg Pro Ser Gln Glu Asn Phe
65                  70                  75                  80

Ser Leu Ile Leu Glu Leu Ala Thr Pro Ser Gln Thr Ser Val Tyr Phe
                85                  90                  95

Cys Ala Ser Gly Gly Gln Gly Arg Ala Glu Gln Phe Phe Gly Pro Gly
            100                 105                 110

Thr Arg Leu Thr Val Leu Gly Ser Asp Tyr Lys Asp Asp Asp Asp Lys
        115                 120                 125

Arg Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Ser Gly
    130                 135                 140

Ala Gln Gln Gln Val Arg Gln Ser Pro Gln Ser Leu Thr Val Trp Glu
145                 150                 155                 160

Gly Glu Thr Thr Ile Leu Asn Cys Ser Tyr Glu Asp Ser Thr Phe Asp
                165                 170                 175

Tyr Phe Pro Trp Tyr Arg Gln Phe Pro Gly Lys Ser Pro Ala Leu Leu
            180                 185                 190

Ile Ala Ile Ser Leu Val Ser Asn Lys Lys Glu Asp Gly Arg Phe Thr
        195                 200                 205

Ile Phe Phe Asn Lys Arg Glu Lys Lys Leu Ser Leu His Ile Thr Asp
    210                 215                 220

Ser Gln Pro Gly Asp Ser Ala Thr Tyr Phe Cys Ala Ala Thr Gly Ser
225                 230                 235                 240

Phe Asn Lys Leu Thr Phe Gly Ala Gly Thr Arg Leu Ala Val Ser Pro
                245                 250                 255
```

Tyr His His His His His His
260

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 798 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..786

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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AGC TCT CTG GTA CCG CGG GGC TCT AAA GTC TTA CAG ATC CCA AGT CAT      48
Ser Ser Leu Val Pro Arg Gly Ser Lys Val Leu Gln Ile Pro Ser His
265                 270                 275

CAA ATA ATA GAT ATG GGG CAG ATG GTG ACC CTC AAT TGT GAC CCA GTT      96
Gln Ile Ile Asp Met Gly Gln Met Val Thr Leu Asn Cys Asp Pro Val
280                 285                 290                 295

TCT AAT CAC CTA TAT TTT TAT TGG TAT AAA CAG ATT TTA GGA CAG CAG     144
Ser Asn His Leu Tyr Phe Tyr Trp Tyr Lys Gln Ile Leu Gly Gln Gln
                300                 305                 310

ATG GAG TTT CTT GTT AAT TTC TAC AAT GGT AAA TTC ATG GAG AAG TCT     192
Met Glu Phe Leu Val Asn Phe Tyr Asn Gly Lys Phe Met Glu Lys Ser
            315                 320                 325

AAA CTG TTT AAG GAT CAG TTT TCA GTT GAA AGA CCA GAT GGT TCA TAT     240
Lys Leu Phe Lys Asp Gln Phe Ser Val Glu Arg Pro Asp Gly Ser Tyr
        330                 335                 340

TTC ACT CTG AAA ATC CAA CCC ACA GCA CTG GAG GAC TCA GCT GTG TAC     288
Phe Thr Leu Lys Ile Gln Pro Thr Ala Leu Glu Asp Ser Ala Val Tyr
    345                 350                 355

TTC TGT GCC AGC AGC CCG GAC ACA AAC TAT GCT GAG CAG TTC TTC GGA     336
Phe Cys Ala Ser Ser Pro Asp Thr Asn Tyr Ala Glu Gln Phe Phe Gly
360                 365                 370                 375

CCA GGG ACA CGA CTC ACC GTC CTA GGA TCC GAC TAC AAG GAC GAC GAT     384
Pro Gly Thr Arg Leu Thr Val Leu Gly Ser Asp Tyr Lys Asp Asp Asp
                380                 385                 390

GAC AAG AGA TCC GGT GGT GGT GGT TCC GGA GGT GGT GGT TCT GGT GGT     432
Asp Lys Arg Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
            395                 400                 405

TCT GGC GCC GGA GAT CAG GTG GAG CAG AGT CCT TCA GCC CTG AGC CTC     480
Ser Gly Ala Gly Asp Gln Val Glu Gln Ser Pro Ser Ala Leu Ser Leu
        410                 415                 420
```

```
CAC GAG GGA ACC GAT TCT GCT CTG AGA TGC AAT TTT ACT ACC ACC ATG        528
His Glu Gly Thr Asp Ser Ala Leu Arg Cys Asn Phe Thr Thr Thr Met
    425                 430                 435

AGG GCT GTG CAG TGG TTC CGA AAG AAT TCC AGA GGC AGC CTC ATC AAT        576
Arg Ala Val Gln Trp Phe Arg Lys Asn Ser Arg Gly Ser Leu Ile Asn
440                 445                 450                 455

CTG TTC TAC TTG GCT TCA GGA ACA AAG GAG AAT GGG AGG CTA AAG TCA        624
Leu Phe Tyr Leu Ala Ser Gly Thr Lys Glu Asn Gly Arg Leu Lys Ser
                460                 465                 470

GCA TTT GAT TCT AAG GAG CGC TAC AGC ACC CTG CAC ATC AGG GAT GCC        672
Ala Phe Asp Ser Lys Glu Arg Tyr Ser Thr Leu His Ile Arg Asp Ala
            475                 480                 485

CAG CGG GAG GAC TCA GGC ACT TAC TTC TGT GCT GCT GAG GCA ACT TCA        720
Gln Arg Glu Asp Ser Gly Thr Tyr Phe Cys Ala Ala Glu Ala Thr Ser
        490                 495                 500

AGT GGC CAG AAG CTG GTT TTT GGC CAG GGG ACC ATA TTA AAG GTG TAC        768
Ser Gly Gln Lys Leu Val Phe Gly Gln Gly Thr Ile Leu Lys Val Tyr
    505                 510                 515

CAT CAC CAT CAC CAT CAC TAATGAAAGC TT                                  798
His His His His His His
520                 525
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 262 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Ser Ser Leu Val Pro Arg Gly Ser Lys Val Leu Gln Ile Pro Ser His
  1               5                  10                  15

Gln Ile Ile Asp Met Gly Gln Met Val Thr Leu Asn Cys Asp Pro Val
            20                  25                  30

Ser Asn His Leu Tyr Phe Tyr Trp Tyr Lys Gln Ile Leu Gly Gln Gln
        35                  40                  45

Met Glu Phe Leu Val Asn Phe Tyr Asn Gly Lys Phe Met Glu Lys Ser
    50                  55                  60

Lys Leu Phe Lys Asp Gln Phe Ser Val Glu Arg Pro Asp Gly Ser Tyr
65                  70                  75                  80

Phe Thr Leu Lys Ile Gln Pro Thr Ala Leu Glu Asp Ser Ala Val Tyr
                85                  90                  95

Phe Cys Ala Ser Ser Pro Asp Thr Asn Tyr Ala Glu Gln Phe Phe Gly
            100                 105                 110
```

Pro Gly Thr Arg Leu Thr Val Leu Gly Ser Asp Tyr Lys Asp Asp Asp
115 120 125

Asp Lys Arg Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
130 135 140

Ser Gly Ala Gly Asp Gln Val Glu Gln Ser Pro Ser Ala Leu Ser Leu
145 150 155 160

His Glu Gly Thr Asp Ser Ala Leu Arg Cys Asn Phe Thr Thr Thr Met
165 170 175

Arg Ala Val Gln Trp Phe Arg Lys Asn Ser Arg Gly Ser Leu Ile Asn
180 185 190

Leu Phe Tyr Leu Ala Ser Gly Thr Lys Glu Asn Gly Arg Leu Lys Ser
195 200 205

Ala Phe Asp Ser Lys Glu Arg Tyr Ser Thr Leu His Ile Arg Asp Ala
210 215 220

Gln Arg Glu Asp Ser Gly Thr Tyr Phe Cys Ala Ala Glu Ala Thr Ser
225 230 235 240

Ser Gly Gln Lys Leu Val Phe Gly Gln Gly Thr Ile Leu Lys Val Tyr
245 250 255

His His His His His His
260

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ile Glu Gly Arg
1

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Val Pro Arg Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Cys Cys Cys Cys Ala Ala Gly Cys Thr Thr Cys Ala Ala Thr Thr Ala
1 5 10 15

Ala Thr Gly Gly Thr Gly Ala Thr Gly Gly Thr Gly Ala Thr Gly Gly
20 25 30

Thr Gly Ala Thr Ala Thr Gly Gly Gly Gly Ala Cys Ala Cys Ala Gly
35 40 45

Cys Cys Ala Gly Thr Cys Thr Gly Gly Thr Cys Cys Cys
50 55 60

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Cys Gly Ala Ala Thr Thr Cys Ala Gly Gly Cys Gly Cys Cys Cys Ala
1 5 10 15

Gly Cys Ala Gly Cys Ala Ala Gly Thr Gly Ala Gly Ala Cys Ala Ala
20 25 30

Ala Gly Thr Cys Cys Cys Cys
35

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Cys Cys Cys Ala Ala Gly Cys Thr Thr Thr Cys Ala Thr Thr Ala Gly
1 5 10 15

Thr Gly Ala Thr Gly Gly Thr Gly Ala Thr Gly Gly Thr Gly Ala Thr
20 25 30

Gly Gly Thr Ala Cys Ala Cys Cys Thr Thr Thr Ala Ala Thr Ala Thr
35 40 45

Gly Gly Thr Cys Cys Cys Cys Thr Gly Gly Cys Cys
50 55 60

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Gly Ala Gly Ala Thr Cys Thr Ala Thr Gly Ala Gly Cys Thr Cys
1 5 10 15

Thr Cys Thr Gly Gly Thr Ala Cys Cys Gly Cys Gly Gly Gly Gly Cys
20 25 30

Thr Cys Thr Ala Ala Ala Gly Thr Cys Thr Thr Ala Cys Ala Gly Ala
35 40 45

Thr Cys Cys Cys Ala Ala Gly Thr
50 55

CLAIMS

What is claimed is:

1. A fusion protein, comprising a carrier protein connected by a peptide tether to a single chain T cell receptor, wherein the single chain T cell receptor comprises a Vα segment connected by a peptide linker to a Vβ segment.

2. The fusion protein of Claim 1, wherein the Vβ segment is connected by the peptide linker to the Vα segment such that the linker joins the carboxy terminus of the Vβ segment to the amino terminus of the Vα segment.

3. The fusion protein of Claim 1, wherein the Vβ segment is connected by the peptide linker to the Vα segment such that the linker joins the amino terminus of the Vβ segment to the carboxy terminus of the Vα segment.

4. The fusion protein of Claim 1, wherein the carrier protein is maltose binding protein.

5. The fusion protein of Claim 1, wherein the carrier protein connected by the peptide tether to the single chain T cell receptor molecule such that the peptide tether connects the carboxy terminus of the carrier protein to the amino terminus of the single chain T cell receptor molecule.

6. The fusion protein of Claim 1, wherein the Vα and Vβ segments are isolated from D10 T cells.

7. The fusion protein of Claim 1, wherein the Vα and Vβ segments are isolated from B10 T cells.

8. A soluble single chain T cell receptor, comprising a Vα segment connected by a peptide linker to a Vβ segment.

9. The single chain T cell receptor of Claim 8, wherein the Vβ segment is connected by the peptide linker to the Vα segment such that the linker joins the carboxy terminus of the Vβ segment to the amino terminus of the Vα segment.

10. The single chain T cell receptor of Claim 8, wherein the Vβ segment is connected by the peptide linker to the Vα segment such that the linker joins the amino terminus of the Vβ segment to the carboxy terminus of the Vα segment.

11. A DNA molecule comprising a sequence encoding a carrier protein connected by a peptide tether to a single chain T cell receptor, wherein the single chain T cell receptor comprises a Vα segment connected by a peptide linker to a Vβ segment.

12. The DNA molecule of Claim 11, wherein the Vβ gene is connected by a sequence encoding the peptide linker to the 5′ end of the Vα segment.

13. The DNA molecule of Claim 11, wherein the Vα gene is connected by a sequence encoding the peptide linker to the 5′ end of the Vβ segment.

14. The DNA molecule of Claim 11, wherein the sequence encoding the carrier protein is the gene encoding maltose binding protein.

15. The DNA molecule of Claim 11, wherein the genes encoding the Vα and Vβ segments are derived from D10 T cells.

16. The DNA molecule of Claim 11, wherein the genes encoding the Vα and Vβ segments are derived from B10 T cells.

17. An expression vector comprising a DNA molecule, the DNA molecule comprising a sequence encoding a carrier protein connected by a peptide tether to a single chain T cell receptor, wherein the single chain T cell receptor comprises a Vα segment connected by a peptide linker to a Vβ segment.

18. A host cell comprising an expression vector, the expression vector comprising a DNA molecule, the DNA molecule comprising a sequence encoding a carrier protein connected by a peptide tether to a single chain T cell receptor, wherein the single chain T cell receptor comprises a Vα segment connected by a peptide linker to a Vβ segment.

19. A method of isolating and purifying a fusion protein comprising a single chain T cell receptor protein, comprising the steps of:

a) subjecting a solution comprising the fusion protein to a first cycle amylose affinity chromatography, resulting in amylose purified fusion protein produced by the amylose affinity chromatography;

b) subjecting the amylose purified fusion protein to denaturation, and nickel affinity chromatography under denaturing conditions, followed by refolding of the bound fusion protein by applying

a buffer solution gradient, and eluting the protein, resulting in nickel column purified refolded, fusion protein;

c) subjecting the nickel column purified, refolded fusion protein to anticlonotypic immunoaffinity chromatography, resulting in immunoaffinity purified fusion protein;

d) subjecting the immunoaffinity purified fusion protein to size exclusion chromatography, resulting in size exclusion purified fusion protein; and

e) subjecting the size exclusion purified fusion protein to a second cycle of amylose affinity chromatography, thereby resulting in isolated and purified fusion protein.

20. The method of Claim 19, further comprising the steps of:

f) subjecting the isolated and purified fusion protein to thrombin digestion, and collecting the single chain T cell receptor protein produced by the thrombin digestion; and

g) subjecting the single chain T cell receptor protein to nickel affinity chromatography.

21. An antibody to a single chain T cell receptor, the single chain T cell receptor comprising a Vα segment connected by a peptide linker to a Vβ segment.

22. The antibody of Claim 21, wherein the antibody is linked to an agent selected from the group consisting of: cytotoxic drugs, toxins, enzymes, and radioactive substances.

23. The antibody of Claim 21, wherein the antibody is a polyclonal antibody.

24. The antibody of Claim 21, wherein the antibody is a monoclonal antibody.

25 The antibody of Claim 24, wherein the antibody is 3E9G2.

26. A method of depleting pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a single chain T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

27. A method of inhibiting the activation of pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a single chain T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

28. An assay for identifying agents that inhibit the interaction of T cell receptor with a complex formed between an Major Histocompatibility Complex/Human Leukocyte Antigen Complex (MHC/HLA) molecule and an antigenic peptide of interest, comprising the steps of:

a) incubating a sample of isolated and purified single chain T cell receptor with the MHC/HLA molecule and antigenic peptide of interest, to allow the single chain T cell receptor to interact with the MHC/HLA molecule and antigenic peptide;

b) incubating a sample of isolated and purified single chain T cell receptor with the MHC/HLA molecule and antigenic peptide of interest, and the agent to be tested, to allow the single chain T cell receptor to interact with the MHC/HLA molecule and antigenic peptide;

c) evaluating the level of interaction between the single chain T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide in the presence of the agent to be tested and in the absence of the agent to be tested,

wherein less interaction between the single chain T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide in the presence of the agent to be tested than in the absence of the agent to be tested, is indicative that the agent inhibits the interaction between T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide.

29. An agent identified by the assay of Claim 28.

30. An assay for identifying agents that inhibit the interaction of T cell receptor with a T cell receptor specific antibody of interest, comprising the steps of:

a) incubating a sample of isolated and purified single chain T cell receptor with the T cell receptor specific antibody, to allow the single chain T cell receptor to interact with the T cell receptor antibody;

b) incubating a sample of isolated and purified single chain T cell receptor with the T cell receptor specific antibody and the agent to be

tested, to allow the single chain T cell receptor to interact with the T cell receptor specific antibody;

c) evaluating the level of interaction between the single chain T cell receptor and the T cell receptor specific antibody in the presence of the agent to be tested and in the absence of the agent to be tested,

wherein less interaction between the single chain T cell receptor and the T cell receptor specific antibody in the presence of the agent to be tested than in the absence of the agent to be tested, is indicative that the agent inhibits the interaction between T cell receptor and the T cell receptor specific antibody.

31. An agent identified by the assay of Claim 30.

32. An assay for detecting the presence of pathogenic T cells, comprising the steps of:

a) incubating a sample of lymphocytes with an antibody to a single chain T cell receptor that has a native-like conformation of T cell receptors present on a pathogenic T cell, thereby generating a test sample; and

b) evaluating the test sample for the presence of interaction between the lymphocytes and the antibody,

wherein the presence of interaction between the lymphocytes and the antibody is indicative of the presence of pathogenic T cells.

33. A method of reducing the activation of pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of a single

chain T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

34. A method of immunizing a mammal against T cell receptor antigenic structures on the surface of pathogenic T cells, comprising administering to the mammal an effective amount of a single chain T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

35. The method of Claim 30, wherein the single chain T cell receptor is denatured.


FIGURE 1

```
ATGAAAATAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGACGATGATGTTT
 M  K  I  K  T  G  A  R  I  L  A  L  S  A  L  T  T  M  M  F

TCCGCCTCGGCTCTCGCCAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGAT
 S  A  S  A  L  A  K  I  E  E  G  K  L  V  I  W  I  N  G  D

AAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATTAAA
 K  G  Y  N  G  L  A  E  V  G  K  K  F  E  K  D  T  G  I  K

GTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGC
 V  T  V  E  H  P  D  K  L  E  E  K  F  P  Q  V  A  A  T  G

GATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGC
 D  G  P  D  I  I  F  W  A  H  D  R  F  G  G  Y  A  Q  S  G

CTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGG
 L  L  A  E  I  T  P  D  K  A  F  Q  D  K  L  Y  P  F  T  W

GATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCG
 D  A  V  R  Y  N  G  K  L  I  A  Y  P  I  A  V  E  A  L  S

CTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCG
 L  I  Y  N  K  D  L  L  P  N  P  P  K  T  W  E  E  I  P  A

CTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCG
 L  D  K  E  L  K  A  K  G  K  S  A  L  M  F  N  L  Q  E  P

TACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAACGGC
 Y  F  T  W  P  L  I  A  A  D  G  G  Y  A  F  K  Y  E  N  G

AAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTC
 K  Y  K  I  K  D  V  G  V  D  N  A  G  A  K  A  G  L  T  F

CTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATCGCAGAA
 L  V  D  L  I  K  N  K  H  M  N  A  D  T  D  Y  S  I  A  E

GCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAAC
 A  A  F  N  K  G  E  T  A  M  T  I  N  G  P  W  A  W  S  M

ATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCA
 I  D  T  S  K  V  N  Y  G  V  T  V  L  P  T  F  K  G  Q  P

TCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACAAAGAG
 S  K  P  F  V  G  V  L  S  A  G  I  N  A  A  S  P  N  K  E

CTGGCGAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCGGTTAAT
 L  A  K  E  F  L  E  N  Y  L  L  T  D  E  G  L  E  A  V  N

AAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCGAAAGAT
 K  D  K  P  L  G  A  V  A  L  K  S  Y  E  E  E  L  A  K  D

CCACGTATTGCCGCCACCATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCG
 P  R  I  A  A  T  M  E  N  A  Q  K  G  E  I  M  P  N  I  P

CAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGT
 Q  M  S  A  F  W  Y  A  V  R  T  A  V  I  N  A  A  S  G  R

                                                  Sac I
CAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGCTC
 Q  T  V  D  E  A  L  K  D  A  Q  T  N  S  S
```

FIGURE 2

Sac I
GAGCTCTCTGGTACCGCGG
S S L V P R

GGCTCTGCAGTCTCCCAAAGCCCAAGAAACAAGGTGGCAGTAACAGGAGGAAAGGTGACA
G S A V S Q S P R N K V A V T G G K V T

TTGAGCTGTAATCAGACTAATAACCACAACAACATGTACTGGTATCGGCAGGACACGGGG
L S C N Q T N N H N N M Y W Y R Q D T G

CATGGGCTGAGGCTGATCCATTATTCATATGGTGCTGGCAGCACTGAGAAAGGAGATATC
H G L R L I H Y S Y G A G S T E K G D I

CCTGATGGATACAAGGCCTCCAGACCAAGCCAAGAGAACTTCTCCCTCATTCTGGAGTTG
P D G Y K A S R P S Q E N F S L I L E L

GCTACCCCCTCTCAGACATCAGTGTACTTCTGTGCCAGCGGGGGACAGGGGCGGGCTGAG
A T P S Q T S V Y F C A S G G Q G R A E

Bam HI
CAGTTCTTCGGACCAGGGACACGACTCACCGTCCTAGGATCCGACTACAAGGACGACGAT
Q F F G P G T R L T V L G S D Y K D D D

Nar I
GACAAGAGATCCGGTGGTGGTGGTTCCGGAGGTGGTGGTTCTGGTGGTTCTGGCGCCCAG
D K R S G G G G S G G G G S G G S G A Q

CAGCAAGTGAGACAAAGTCCCCAATCTCTGACAGTCTGGGAAGGAGAGACCACAATTCTG
Q Q V R Q S P Q S L T V W E G E T T I L

AACTGCAGTTATGAGGACAGCACTTTTGACTACTTCCCATGGTACCGGCAGTTCCCTGGG
N C S Y E D S T F D Y F P W Y R Q F P G

AAAAGCCCTGCACTCCTGATAGCCATAAGTTTGGTGTCCAATAAAAAGGAAGATGGACGA
K S P A L L I A I S L V S N K K E D G R

TTCACAATCTTCTTCAATAAAAGGGAGAAAAAGCTCTCCTTGCACATCACAGACTCTCAG
F T I F F N K R E K K L S L H I T D S Q

CCTGGAGACTCAGCCACCTACTTCTGTGCAGCAACAGGTAGCTTCAATAAGTTGACCTTT
P G D S A T Y F C A A T G S F N K L T F

HindIII
GGAGCAGGGACCAGACTGGCTGTGTCCCCATATCACCATCACCATCACCATTAATGA
G A G T R L A V S P Y H H H H H H * *

FIGURE 3

Sac I.
AGCTCTCTGGTACCGCGGGGCTCTAAAGTCTTACAGATCCCAAGTCATCAA
S S L V P R G S K V L Q I P S H Q

ATAATAGATATGGGGCAGATGGTGACCCTCAATTGTGACCCAGTTTCTAATCACCTATAT
I I D M G Q M V T L N C D P V S N H L Y

TTTTATTGGTATAAACAGATTTTAGGACAGCAGATGGAGTTTCTTGTTAATTTCTACAAT
F Y W Y K Q I L G Q Q M E F L V N F Y N

GGTAAATTCATGGAGAAGTCTAAACTGTTTAAGGATCAGTTTTCAGTTGAAAGACCAGAT
G K V M E K S K L F K D Q F S V E R P D

GGTTCATATTTCACTCTGAAAATCCAACCCACAGCACTGGAGGACTCAGCTGTGTACTTC
G S Y F T L K I Q P T A L Q D S A V Y F

TGTGCCAGCAGCCCGGACACAAACTATGCTGAGCAGTTCTTCGGACCAGGGACACGACTC
C A S S P D T N Y A E Q F F G P G T R L

Bam HI
ACCGTCCTAGGATCCGACTACAAGGACGACGATGACAAGAGATCCGGTGGTGGTGGTTCC
T V L G S D Y K D D D D K R S G G G G S

Nar I
GGAGGTGGTGGTTCTGGTGGTTCTGGCGCCGGAGATCAGGTGGAGCAGAGTCCTTCAGCC
G G G G S G G S G A G D Q V E Q S P S A

CTGAGCCTCCACGAGGGAACCGATTCTGCTCTGAGATGCAATTTTACTACCACCATGAGG
L S L H E G T G S A L R C N F T T T M R

GCTGTGCAGTGGTTCCGAAAGAATTCCAGAGGCAGCCTCATCAATCTGTTCTACTTGGCT
A V Q W F R K N S R G S L I N L F Y L A

TCAGGAACAAAGGAGAATGGGAGGCTAAAGTCAGCATTTGATTCTAAGGAGCGCTACAGC
S G T K E N G R L K S A F D S K E R Y S

ACCCTGCACATCAGGGATGCCCAGCGGGAGGACTCAGGCACTTACTTCTGTGCTGCTGAG
T L H I R D A Q L E D S G T Y F C A A E

GCAACTTCAAGTGGCCAGAAGCTGGTTTTTGGCCAGGGGACCATATTAAAGGTGTACCAT
A T S S G Q K L V F G N G T I L K V Y H

HindIII
CACCATCACCATCACTAATGAAAGCTT
H H H H H * *

FIGURE 4

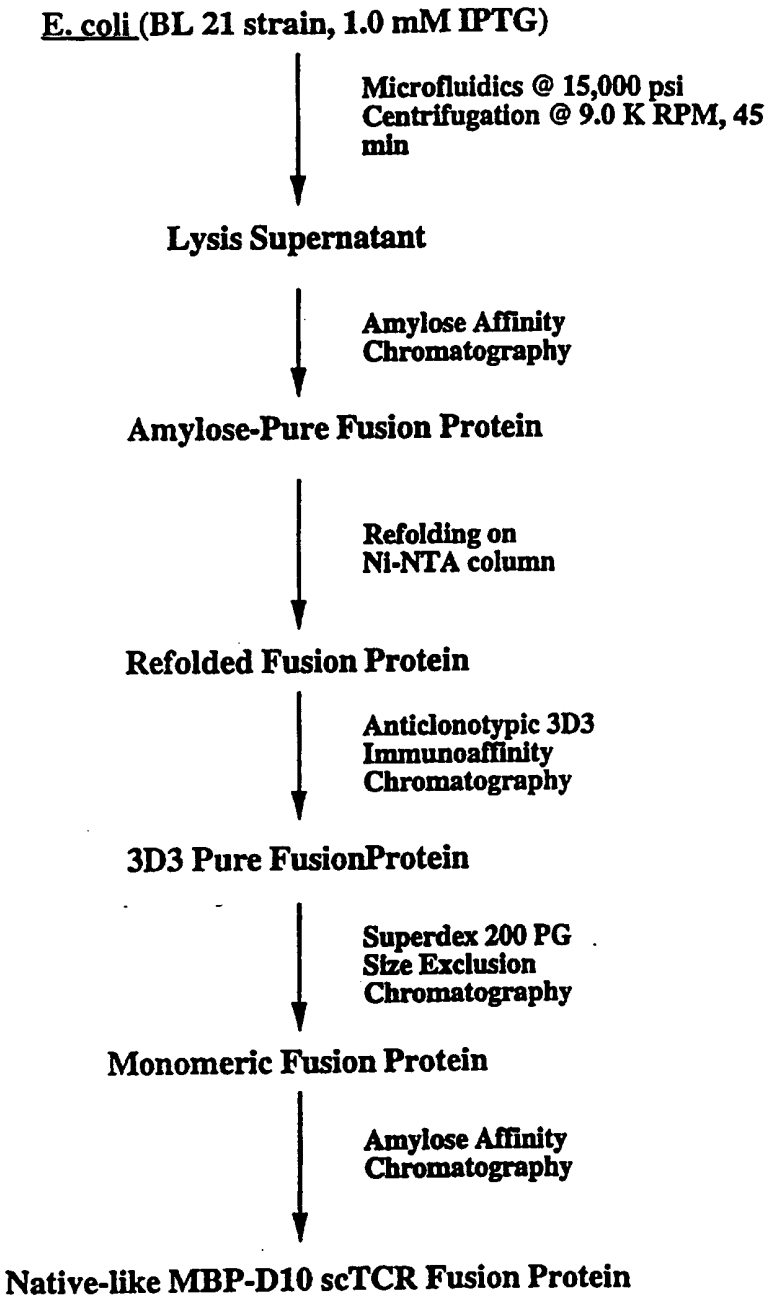
E. coli (BL 21 strain, 1.0 mM IPTG)
Microfluidics @ 15,000 psi
Centrifugation @ 9.0 K RPM, 45 min
Lysis Supernatant
Amylose Affinity Chromatography
Amylose-Pure Fusion Protein
Refolding on Ni-NTA column
Refolded Fusion Protein
Anticlonotypic 3D3 Immunoaffinity Chromatography
3D3 Pure FusionProtein
Superdex 200 PG Size Exclusion Chromatography
Monomeric Fusion Protein
Amylose Affinity Chromatography
Native-like MBP-D10 scTCR Fusion Protein

FIGURE 5

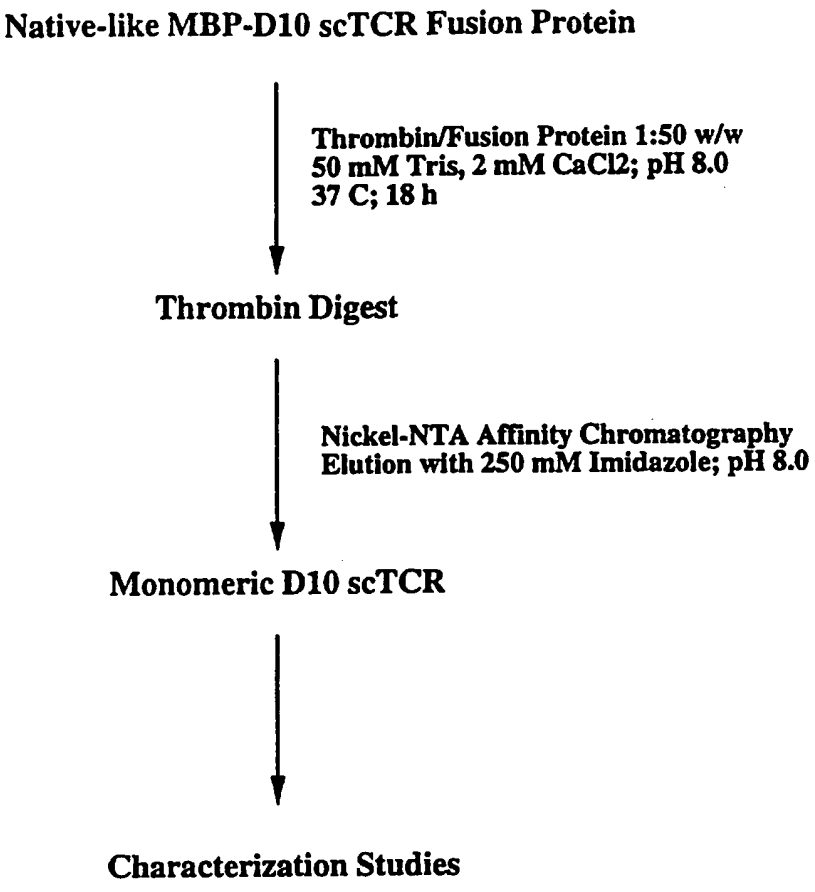
Native-like MBP-D10 scTCR Fusion Protein
Thrombin/Fusion Protein 1:50 w/w
50 mM Tris, 2 mM CaCl2; pH 8.0
37 C; 18 h
Thrombin Digest
Nickel-NTA Affinity Chromatography
Elution with 250 mM Imidazole; pH 8.0
Monomeric D10 scTCR
Characterization Studies

FIGURE 6

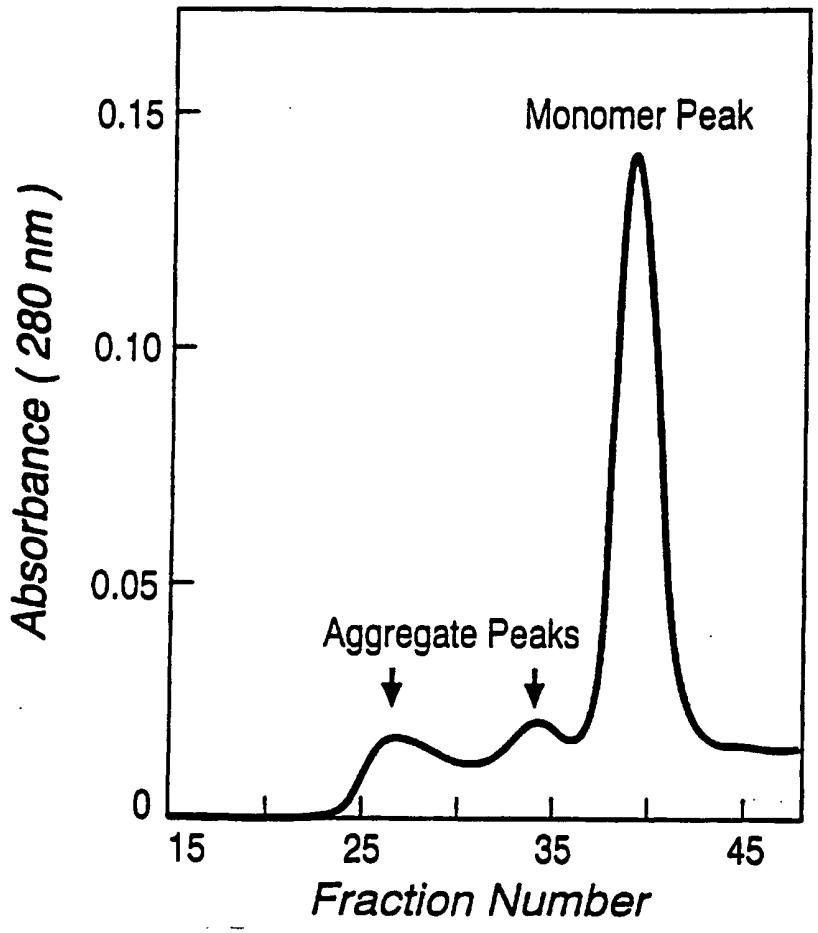
0.15
0.10
0.05
0
Absorbance ( 280 nm )
Monomer Peak
Aggregate Peaks
15
25
35
45
Fraction Number

FIGURE 7

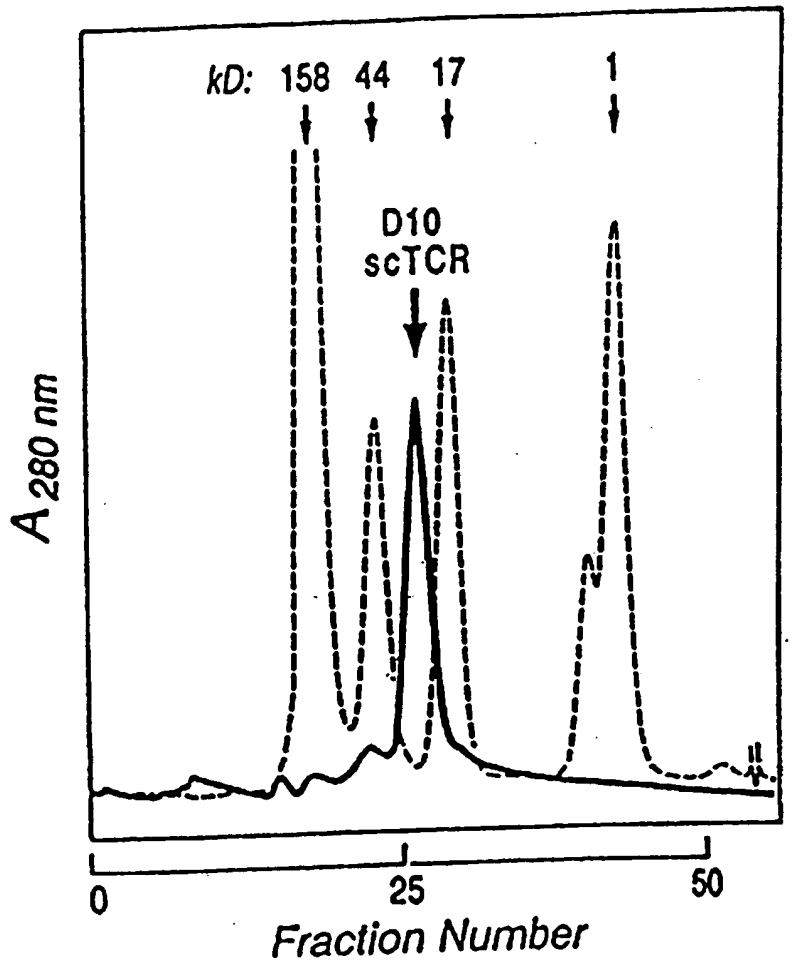


FIGURE 8

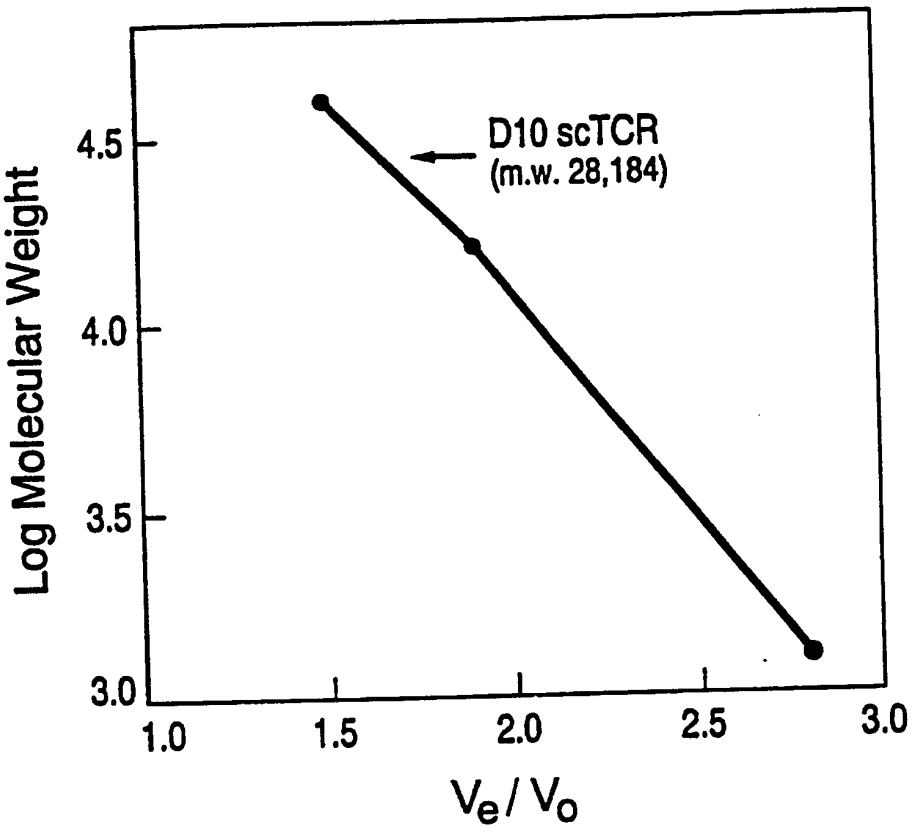
Log Molecular Weight
4.5
4.0
3.5
3.0
1.0
1.5
2.0
2.5
3.0
Ve / Vo
D10 scTCR
(m.w. 28,184)

FIGURE 9

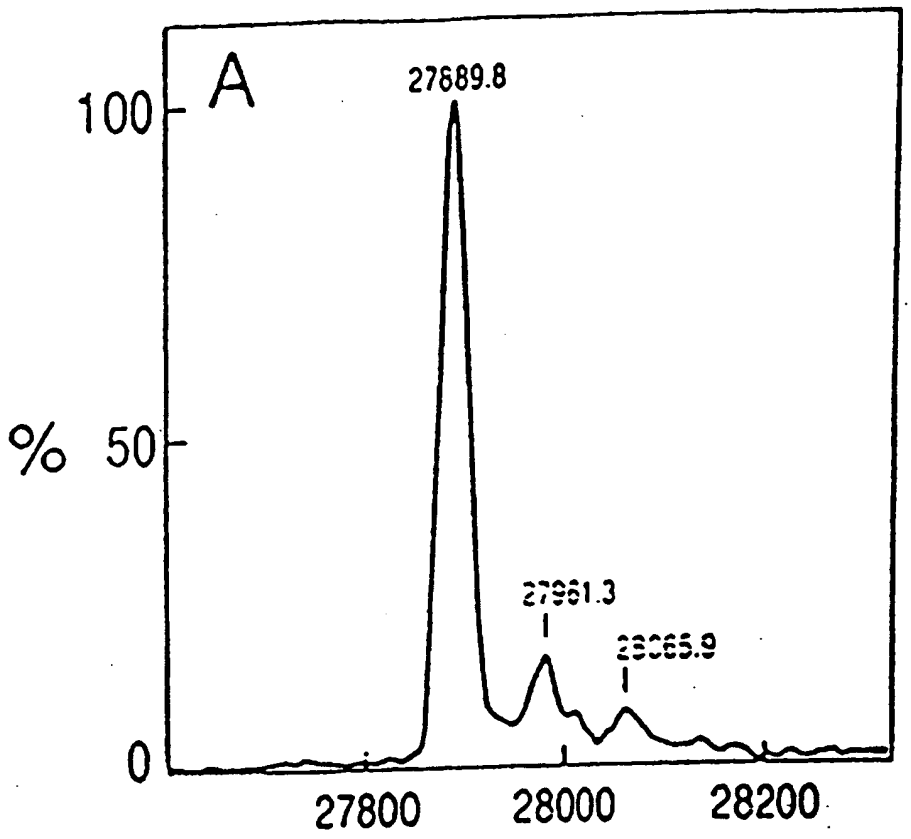


FIGURE 10

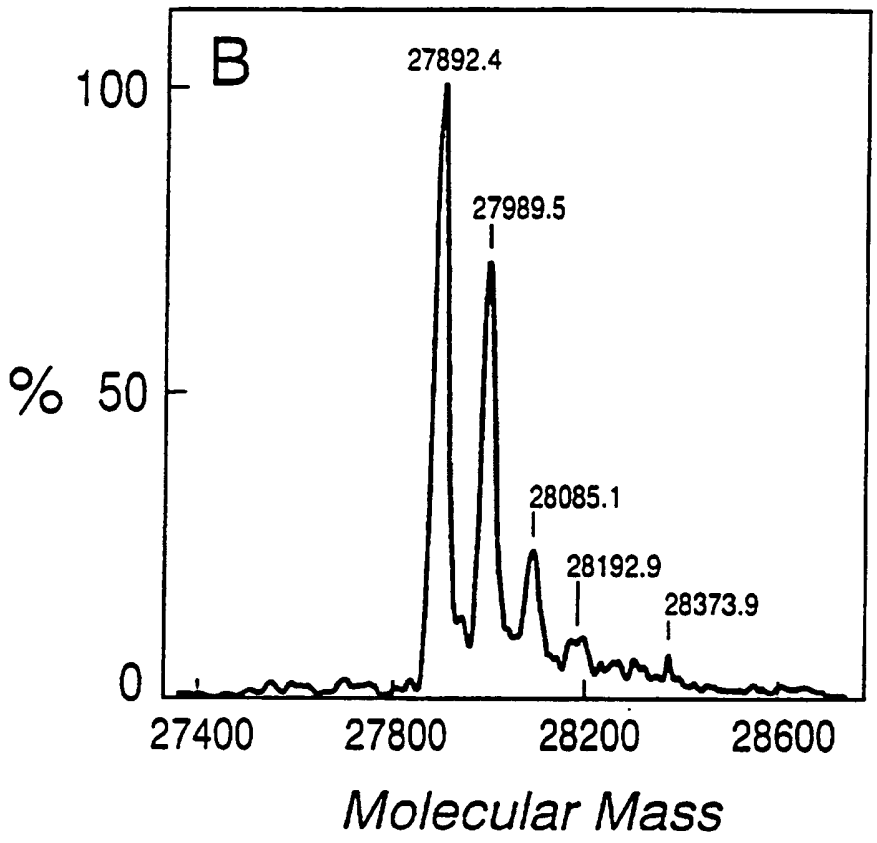
B
100
% 50
0
27892.4
27989.5
28085.1
28192.9
28373.9
27400
27800
28200
28600
Molecular Mass

FIGURE 11

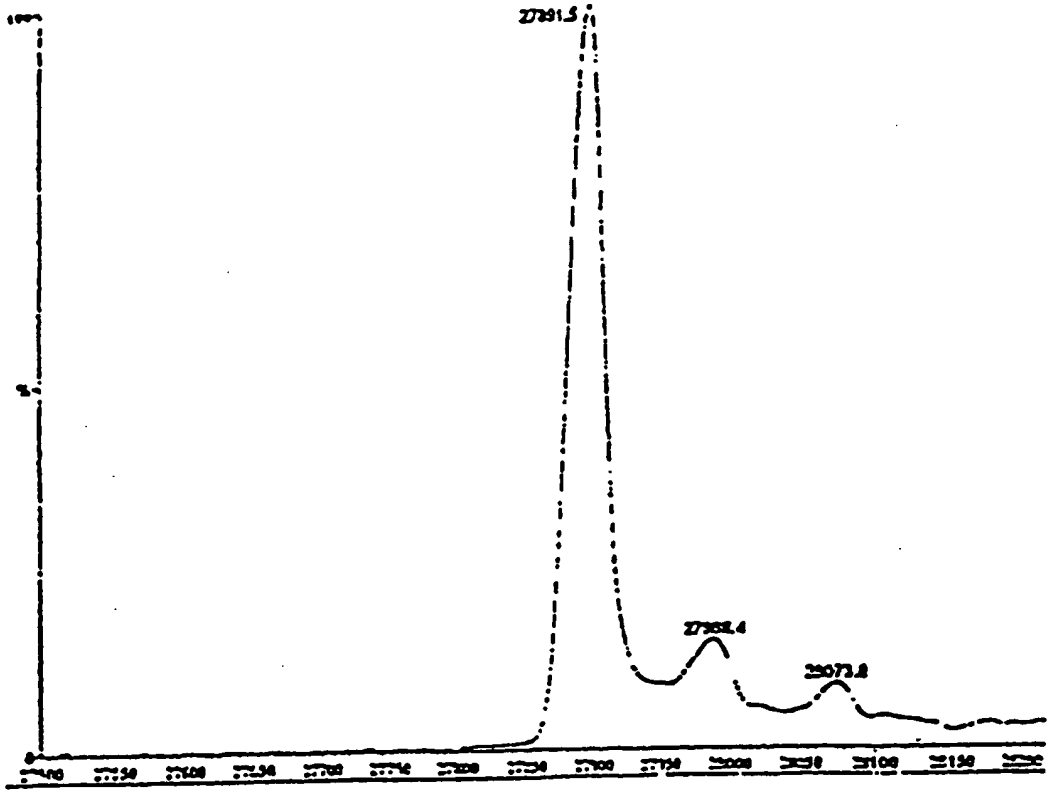


FIGURE 12

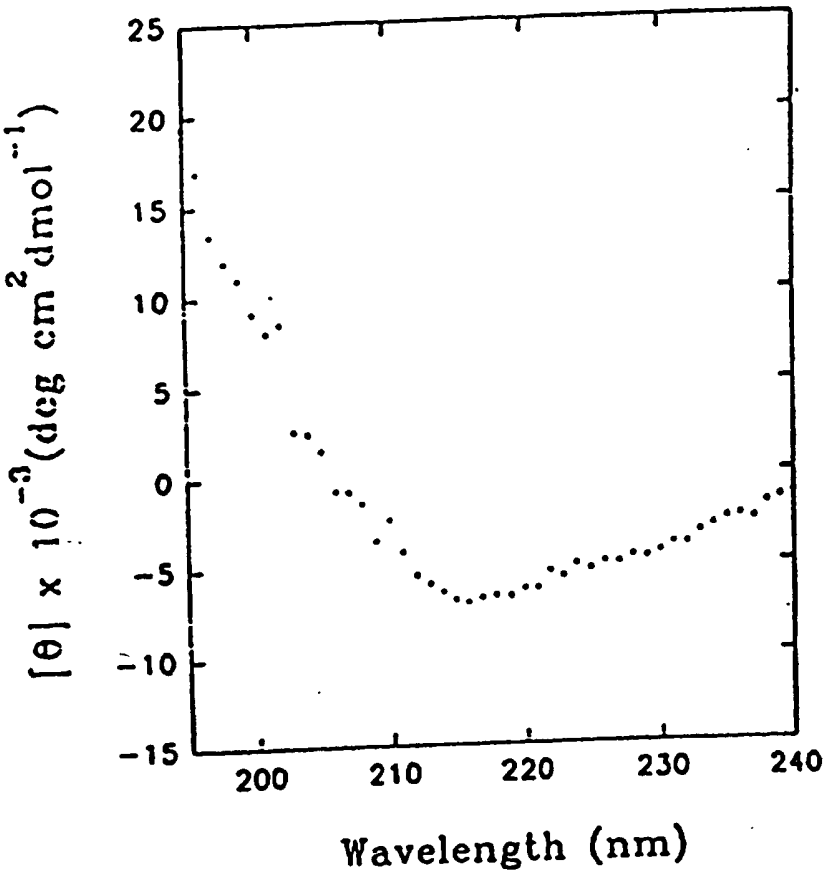
25
20
15
10
5
0
−5
−10
−15
200
210
220
230
240
[θ] x 10^-3 (deg cm^2 dmol^-1)
Wavelength (nm)

FIGURE 13

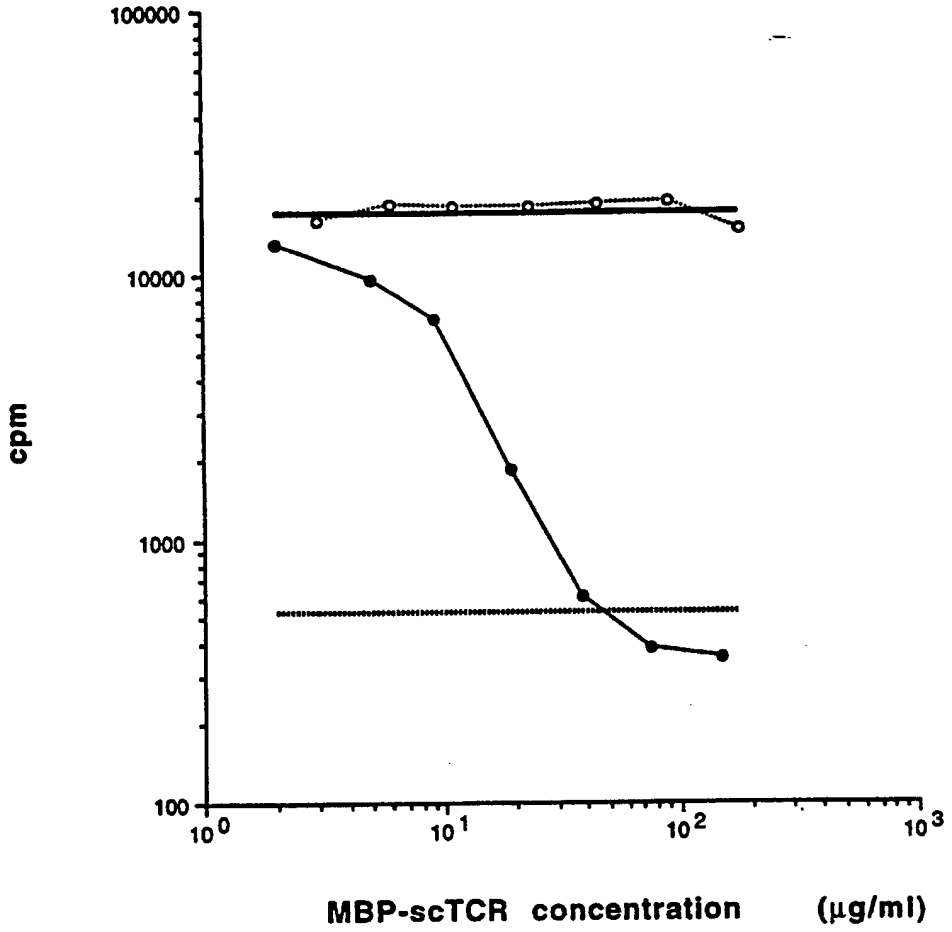
100000
10000
1000
100
cpm
$10^0$
$10^1$
$10^2$
$10^3$
MBP-scTCR concentration (μg/ml)

FIGURE 14

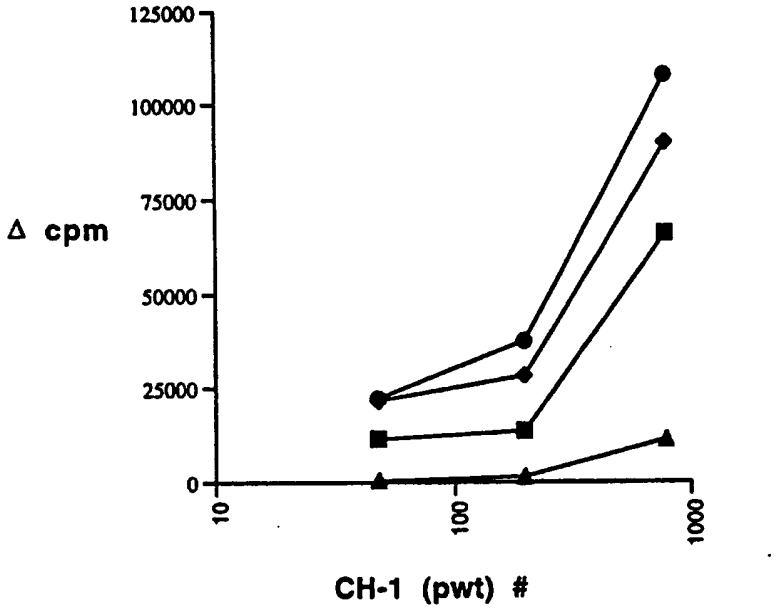


FIGURE 15

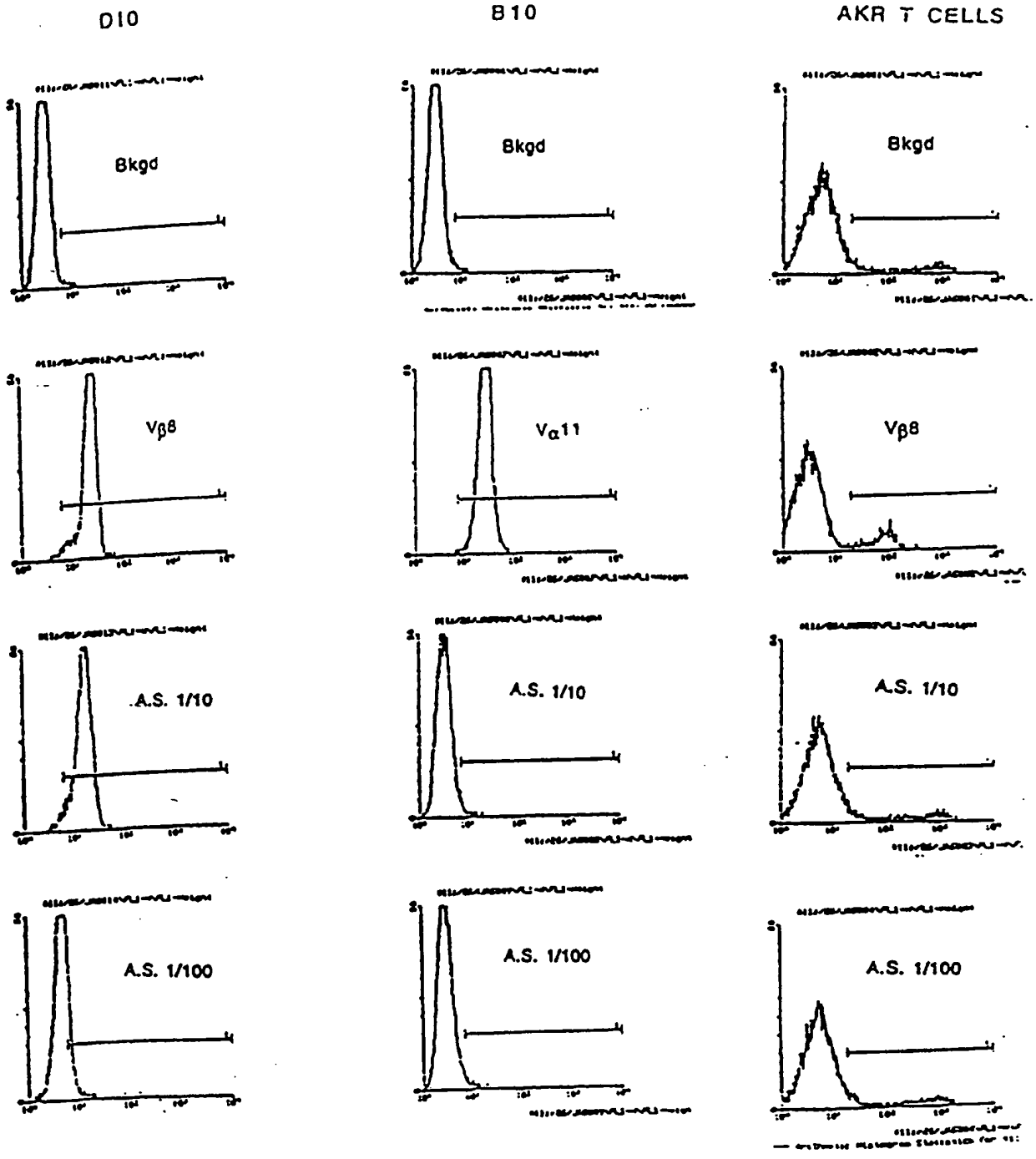
D10
B10
AKR T CELLS
Bkgd
Bkgd
Bkgd
Vβ8
Vα11
Vβ8
A.S. 1/10
A.S. 1/10
A.S. 1/10
A.S. 1/100
A.S. 1/100
A.S. 1/100

FIGURE 16

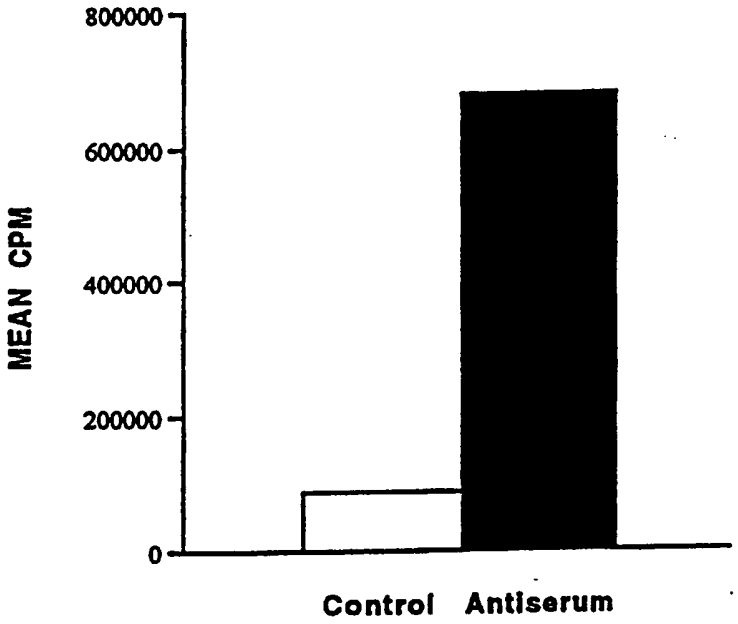


FIGURE 17

## SPECIFICITY OF ANTISERA TO SOLUBLE TCRs

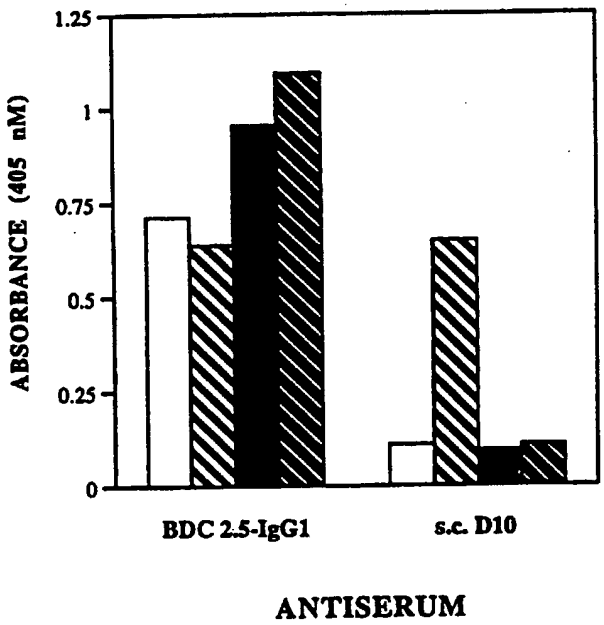


FIGURE 18

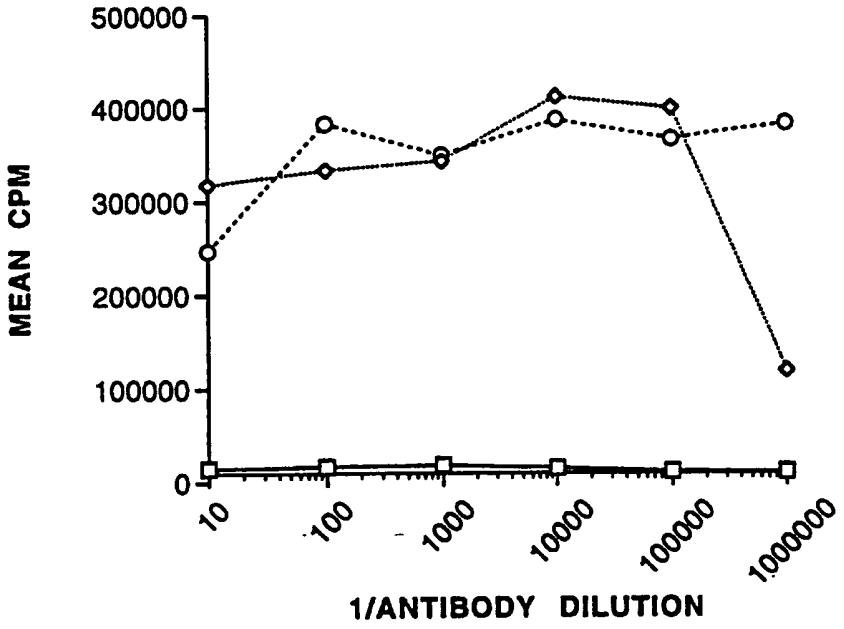
500000
400000
300000
200000
100000
0
MEAN CPM
10
100
1000
10000
100000
1000000
1/ANTIBODY DILUTION

FIGURE 19

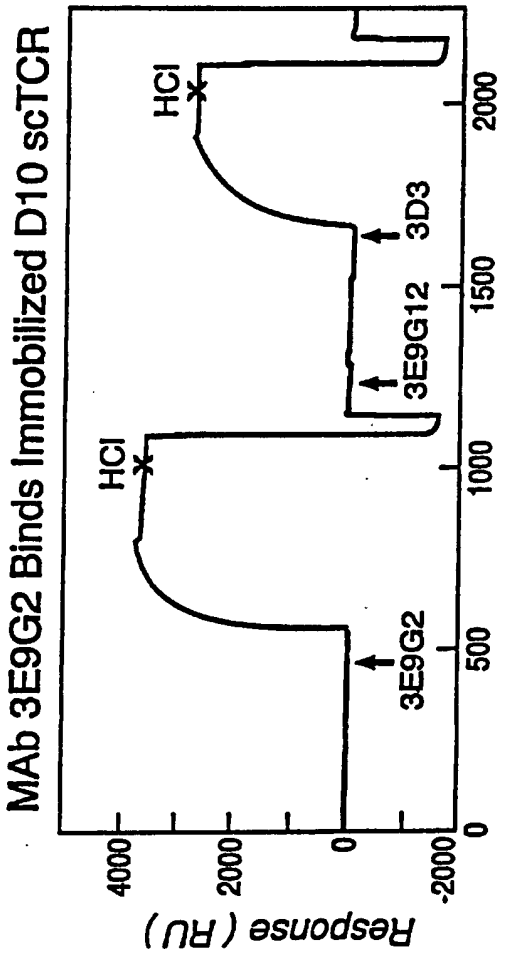
MAb 3E9G2 Binds Immobilized D10 scTCR
HCl
HCl
3E9G2
3E9G12
3D3
Response ( RU )
4000
2000
0
-2000
0
500
1000
1500
2000
Time ( seconds )
Ligand = D10 scTCR (30 µg/ml, pH 5.0) 2500 RU
Analyte (↑) = mAb 3E9G2 (1:100 dln)
3E9G12 (1:100 dln)
3D3 (0.1 mg/ml)

FIGURE 20

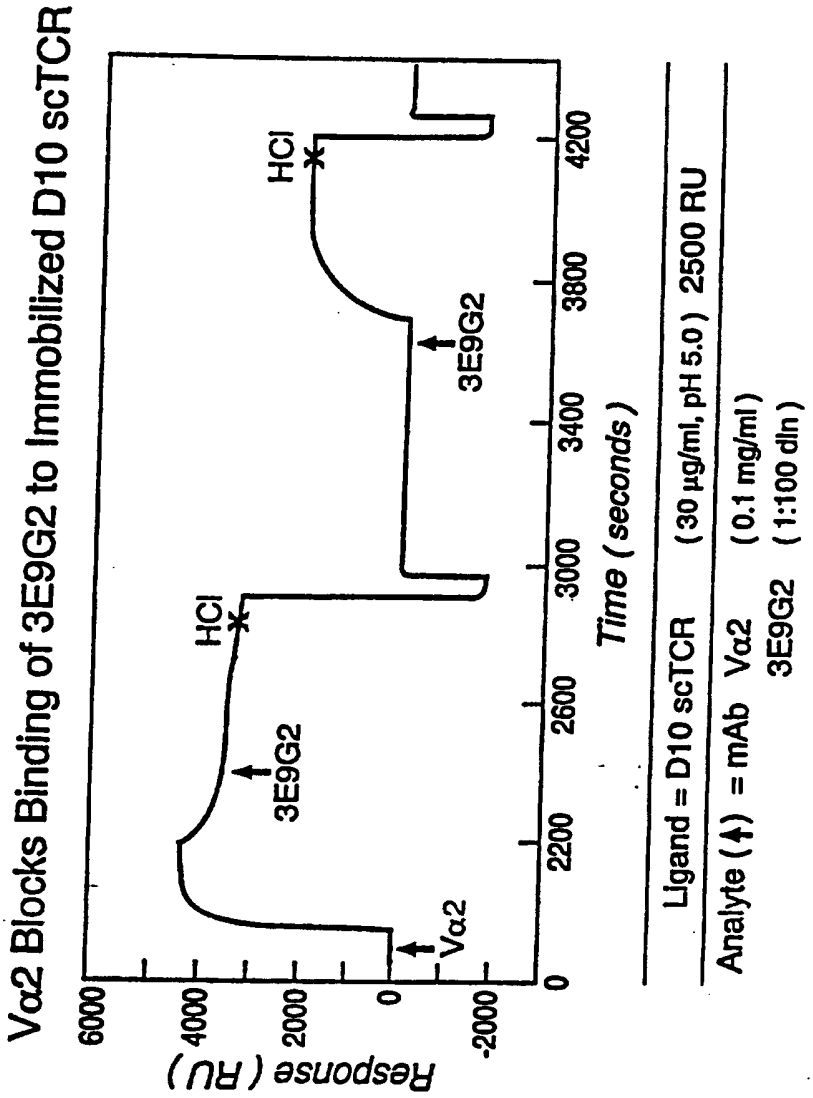
Vα2 Blocks Binding of 3E9G2 to Immobilized D10 scTCR
Response ( RU )
6000
4000
2000
0
-2000
0
2200
2600
3000
3400
3800
4200
Time ( seconds )
Vα2
3E9G2
HCl
3E9G2
HCl
Ligand = D10 scTCR (30 µg/ml, pH 5.0) 2500 RU
Analyte (↑) = mAb Vα2 (0.1 mg/ml)
3E9G2 (1:100 dln)

FIGURE 21

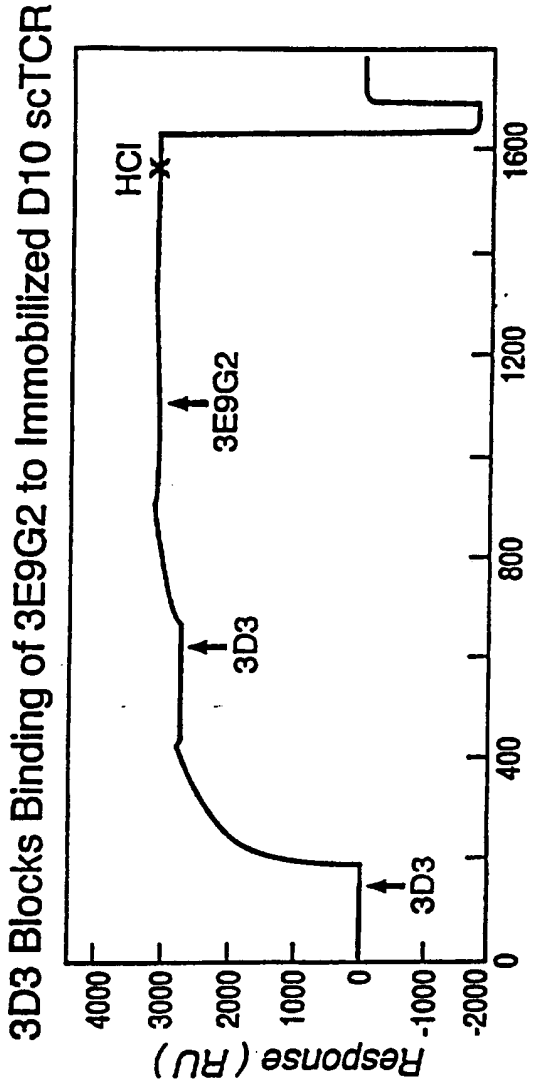
3D3 Blocks Binding of 3E9G2 to Immobilized D10 scTCR
HCl
3E9G2
3D3
3D3
Response ( RU )
4000
3000
2000
1000
0
-1000
-2000
0
400
800
1200
1600
Time ( seconds )
Ligand = D10 scTCR (30 µg/ml, pH 5.0) 2500 RU
Analyte (↑) = mAb 3D3 (0.1 mg/ml)
3E9G2 (1:100 dln)

FIGURE 22

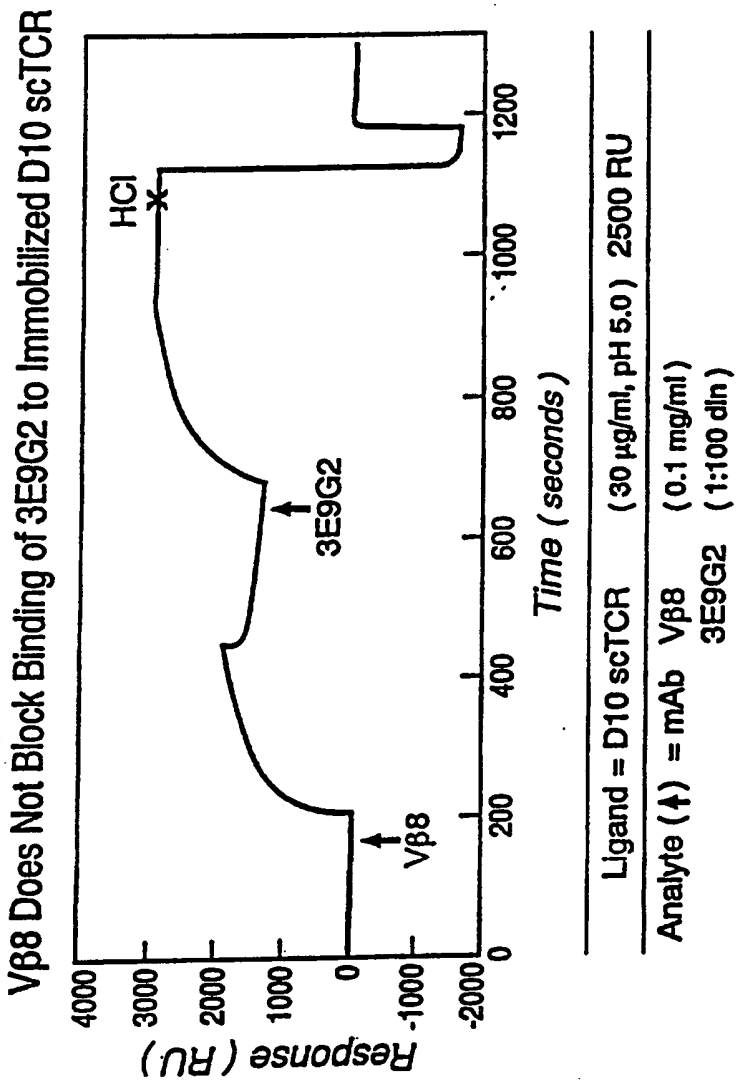
Vβ8 Does Not Block Binding of 3E9G2 to Immobilized D10 scTCR
Response ( RU )
4000
3000
2000
1000
0
-1000
-2000
0
200
400
600
800
1000
1200
Time ( seconds )
HCl
3E9G2
Vβ8
Ligand = D10 scTCR (30 µg/ml, pH 5.0) 2500 RU
Analyte (↑) = mAb Vβ8 (0.1 mg/ml)
3E9G2 (1:100 dln)

FIGURE 23

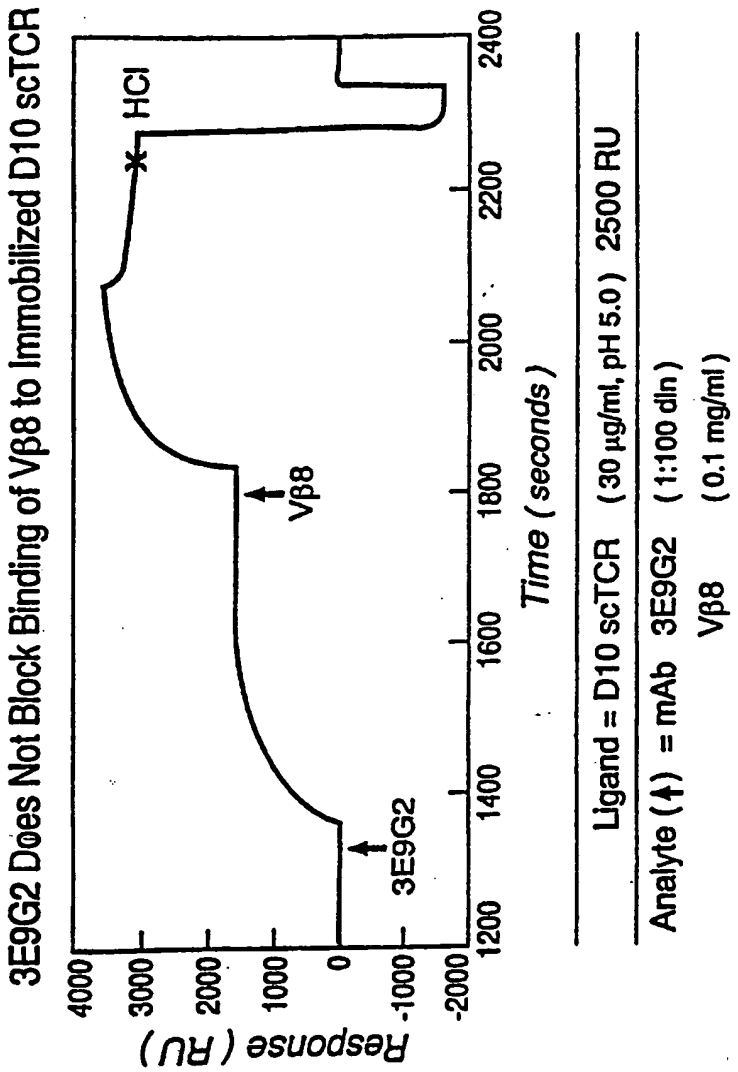
3E9G2 Does Not Block Binding of Vβ8 to Immobilized D10 scTCR
Response ( RU )
4000
3000
2000
1000
0
-1000
-2000
1200
1400
1600
1800
2000
2200
2400
Time ( seconds )
HCl
Vβ8
3E9G2
Ligand = D10 scTCR (30 µg/ml, pH 5.0) 2500 RU
Analyte (↑) = mAb 3E9G2 (1:100 dln)
Vβ8 (0.1 mg/ml)

FIGURE 24

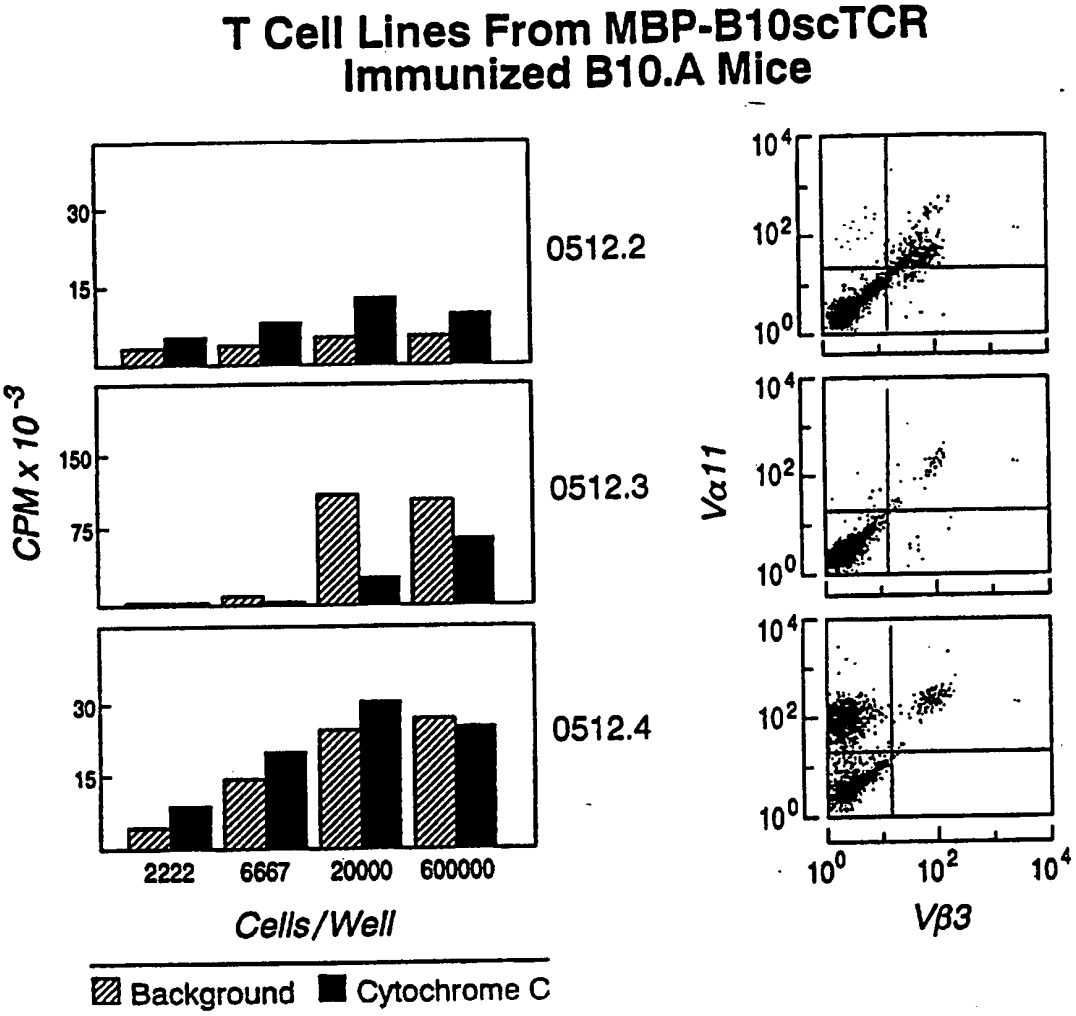
T Cell Lines From MBP-B10scTCR
Immunized B10.A Mice
CPM x 10-3
30
15
0512.2
150
75
0512.3
30
15
0512.4
2222
6667
20000
600000
Cells/Well
Background
Cytochrome C
Vα11
Vβ3
10^4
10^2
10^0

FIGURE 25

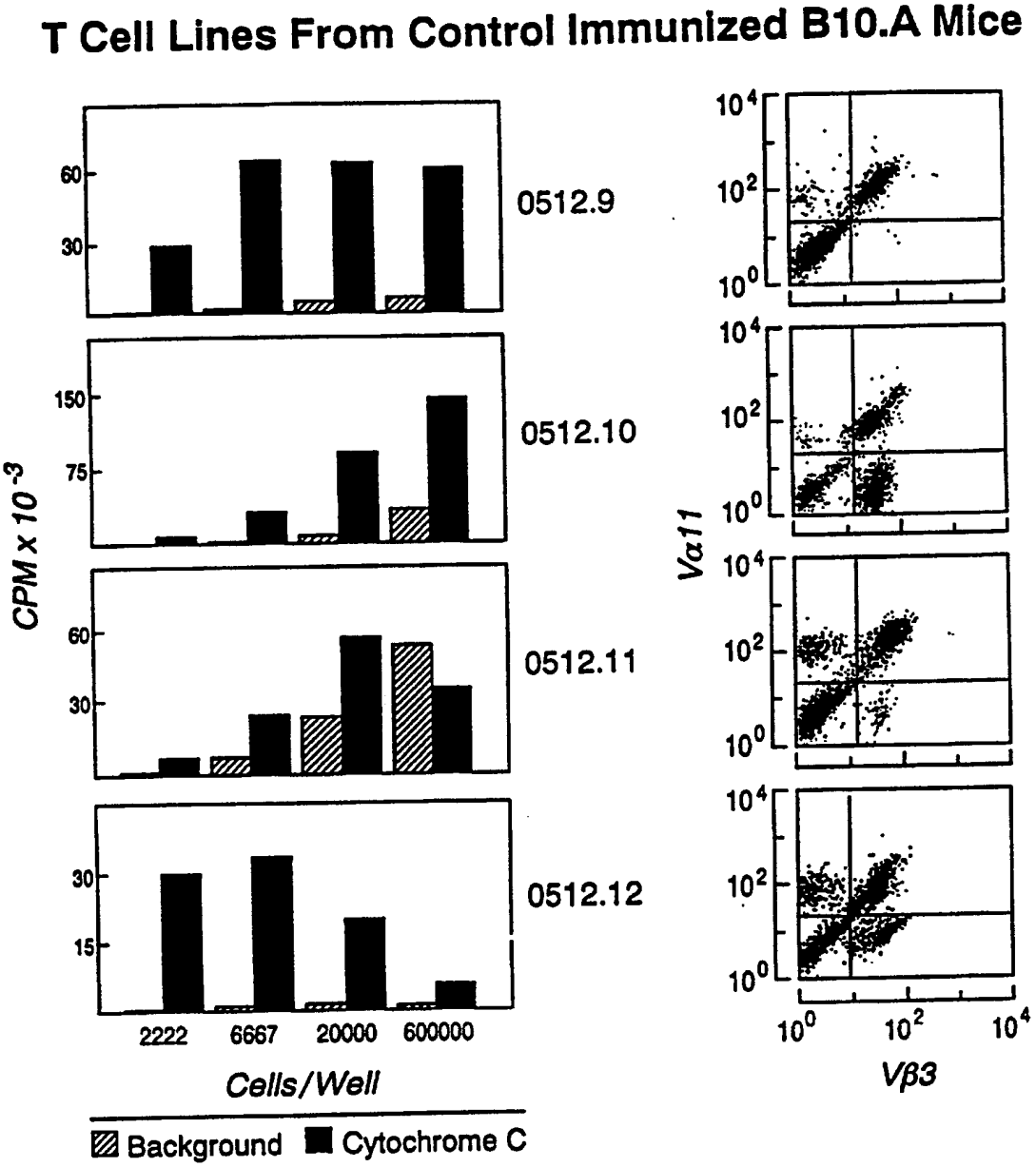
T Cell Lines From Control Immunized B10.A Mice
CPM x 10-3
60
30
0512.9
150
75
0512.10
60
30
0512.11
30
15
0512.12
2222
6667
20000
600000
Cells/Well
Background
Cytochrome C
Vα11
Vβ3
10^4
10^2
10^0

FIGURE 26

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